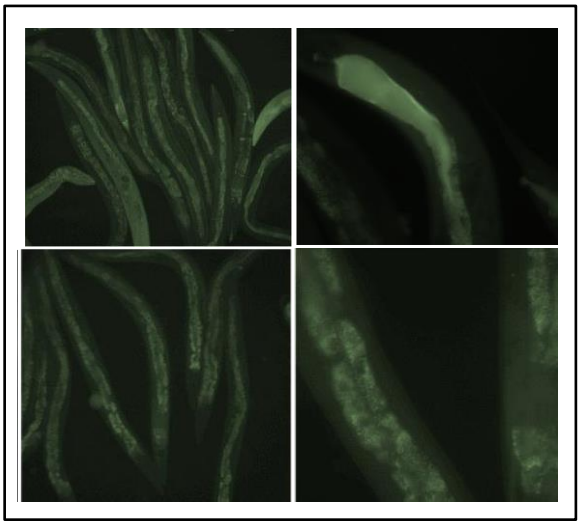
Development of an in-vivo high throughput assay to monitor biofilm development of the pathogenic microorganism *Pseudomonas aeruginosa*, in *C. elegans*.

Martina Ragno



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DECLARATION

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning

Martina Ragno, July 2023

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ABSTRACT

Bacterial biofilms are clusters of bacteria attached to host tissues and abiotic surfaces, such as medical implants, imbedded in a self-generated matrix; this is characterised by extracellular polysaccharides, proteins and eDNA, providing nutrients and protection to the bacteria, which become resistant to both the host's immune system and antimicrobials, therefore becoming extremely difficult to treat and eradicate.

About 80% of recurring chronic microbial diseases in humans are caused by bacterial biofilms, recognition of which has led to an increased attention on identifying new treatments. Particularly, biofilms are difficult to detect in clinical settings, due to lack of biofilm-specific biomarkers. Therefore, further studies are essential to identify markers unique to bacterial biofilms.

In this study, we mainly focused on Quorum Sensing (QS) signalling, a well-known system of communication found in many microbial species, involved in cell density regulation for biofilm formation. Using the biofilm-former *Pseudomonas aeruginosa (P. aeruginosa)*, we targeted QS-related genes, to build a biofilm-specific reporter, to use for in-vivo studies in the nematode *Caenorhabditis elegans (C. elegans).* Particularly, we aimed to create a high-throughput *C. elegans* in-vivo biofilm assay for industrial research and to provide a cost and time-effective protocol for it.

C. elegans is extremely advantageous to address many research questions, particularly due to its transparency, short generation time and low maintenance costs; moreover, it shares more than 80% of human genes and it is colonised by pathogenic bacteria, such as *P. aeruginosa.*

Our results show that *P. aeruginosa* transgenic fluorescent reporters can be employed in a *C. elegans* biofilm infection model, which can provide a non-invasive host's health readout and visualisation of bacterial tissue colonisation; detection of fluorescence signal by biofilm-competent bacteria, as opposed to lack of signal by mutant biofilm-incompetent bacteria, also suggested that *C. elegans* could be used to monitor biofilm progression in the animal.

Further studies will be needed to complement this research, to prove evidence of biofilm detection and to address standardisation of this assay for a possible industrial use; overall, this was a first step which holds promise in the attempt to bridge the gap between in-vivo clinical diagnostics and in-vitro biofilm research.

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ABBREVIATIONS

Abbreviation	Meaning
AHL	N-acylated homoserine lactone
AI	Autoinducer
BHL	N-butyryl-L-homoserine lactone
c-di-GMP	Bis-(3'-5')-cyclic diguanosine monophosphate
C. elegans	Caenorhabditis elegans
CF	Cystic Fibrosis
CFTR regulator	Cystic Fibrosis transmembrane conductance
CFU	Colony Forming Unit
CLSM	Confocal laser scanning microscopy
CV	Crystal Violet
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FLP	Flippase enzyme
GSC	Germline stem cells
HHQ	2-heptyl-3-hydroxy-4-quinolone
L1, L2, L3, L4	Larval stage 1, 2, 3, 4
LB	Luria Bertani broth
MCC	Mucociliary clearance
MCS	Multiple cloning site
MH	Mueller Hinton broth
MVs	Membrane vesicles
NGM	Nematode Growth Media
NPO	4-Nitropyridine-N-oxide
OdDHL	N-(3-oxododecanoyl)-L-homoserine lactone
p-value	Probability
P. aeruginosa	Pseudomonas Aeruginosa
PCR	Polymerase chain reaction ()
PQS	*pseudomonas* quinolone signal ()
QS	Quorum Sensing
QQ	Quorum quenching
SD	Standard deviation
SEM	Scanning electron microscopy
sRNAs	Small RNAs
Tet	Tetracycline
TEM	Transmission electron microscopy
WT	Wild type

CHAPTER 1: INTRODUCTION

1.1 Project overview

Most of recurring chronic microbial diseases in humans are caused by bacterial biofilms (about 80%). (1) A clear example is represented by the opportunistic human pathogen *Pseudomonas aeruginosa (P. aeruginosa)*, which, thanks to its capability to form antibiotic-resistant biofilms, is a persistent pathogen in clinical environments causing deadly infections (1)

Biofilms can form on both abiotic surfaces such as medical devices (e.g. heart valves, catheters, dental units etc) and host tissues (such as lungs, urinary tract, bloodstream etc), making it a big threat to patients, especially immunocompromised people. Antibiotic resistance is one of the major factors contributing to establishment of chronic infection by the biofilm communities. (2) This is the result of the multicellular nature of biofilm and the intensive communication among its cells by Quorum Sensing, allowing genetic up/down regulation in response to external stimuli, leading to formation of complex and well organised structures. (2) These are made of extracellular polymeric substances to shield from the outer environment and adopt many mechanisms, such as employment of eflux pumps, to slow down antimicrobial agents' penetration. (2)

Therefore, alternative strategies are required to eradicate biofilm multi-drug resistance. Particularly, a method needs to be developed to monitor biofilm formation in vivo. So far, current tests are performed in vitro only, in cultured cells, which doesn't give a proper insight into biofilm-host interaction. (3) Developing a new in vivo assay is necessary not only to better understand biofilm formation but also to improve pre-clinical antimicrobial testing, boosting the efficiency of antimicrobial drug development which, up to now, has mainly be focusing on planktonic bacteria. (3)

Caenorhabditis elegans (C. elegans) offers a great potential to develop a non-mammalian in vivo biofilm assay. (4) *C. elegans* is a nematode with short generation time and therefore very

easily cultivated in laboratory settings. (5) It possesses a sequenced genome which makes it very useful in biomedical research. (5) Moreover, it is infected by clinically relevant pathogens such as *P. aeruginosa*, making it a perfect candidate for monitoring of microbial pathogenesis. (6) Finally, this may be easily tracked thanks to the nematode transparency and upon employment of fluorescent reporters. (7)

The goal of this research is to create a high-throughput *C. elegans* assay for industrial research and to provide a cost and time-effective protocol for it. This will be done by making transgenic bacteria which express orange fluorescence driven by expression of selected biofilm-related genes, monitored using fluorescence microscopy. Furthermore, other *Pseudomonas* reporters, already employed in other researches, will be considered for their possible use in biofilm studies and for the development of an in vivo biofilm assay.

1.1.1 Bacterial biofilms and disease

There has been an increased focus on understanding the role played by bacterial biofilm in disease. (1) In fact, these represent 65% of all microbial infections and 80% of all chronic infections, as revealed by the National Institute of Health (NIH), causing worldwide morbidity. (2)

Being resilient to the host immune system and to antibiotic treatments, biofilms mostly cause chronic infections which can affect the cardiovascular, digestive, integumentary, reproductive, respiratory and the urinary system. (1) Some examples of biofilm induced diseases are Otitis Media, Infective Endocarditis, Bacterial Vaginosis, chronic Rhinosinusitis, Periodontitis and many more could be cited. (1)

1.1.2 Biofilm contamination and disease

Biofilm infections also interest medical and implant devices, such as joint prostheses, mechanical heart valves, catheters, contact lenses etc. (2) These are usually the consequence of fungi colonization such as species of *Candida,* and/or bacteria colonization of the species Streptococci and Staphylococci such as *S. aureus* and *S. epidermidis* and/or gram negative bacteria such as *E. coli*, *P. aeruginosa*, etc.(2)

The opportunistic, gram negative bacterium *P. aeruginosa* accounts for almost 30% of device-related biofilm contaminations. (3) This is an ubiquitous, aerobic, non-spore forming microorganism, causing deadly infections, especially in immune-compromised patients and those affected by severe burns; it is also one of the major causes of nosocomial pneumonia and chronic lung infections in patients with Cystic Fibrosis (CF), characterised by high fatality rates. (4)

1.1.3 *P. aeruginosa* nosocomial acquirement

Exogenous transmission plays the greater role in nosocomial acquirement of *P. aeruginosa*, mainly caused by health care workers and patients' interactions, via contaminated hands, gloves gowns and medical devices/implants. (5) Also, antimicrobial selective pressure within the host, may lead to endogenous acquisition of resistant strains of the gram-negative bacterium. (5) Finally, environmental reservoirs play a role in spread of *P. aeruginosa*, with water sources being the most common sites of colonisation, such as showerheads and sinks, but also door handles, intravenous pumps, ventilators etc. (5)

1.1.4 *P. aeruginosa* and Cystic Fibrosis (CF)

CF is a genetic disease characterised by a mutation in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), causing affected people to have thick mucus layers on their airways surfaces; this environment favours bacterial growth and colonisation, often resulting in declined pulmonary functions and mortality.(6) In CF patients, several factors may facilitate *P. aeruginosa* adherence and resistance, such as abnormal composition of airway surface liquid and malfunctioning anti-microbial peptides, increased availability of bacterial receptors, defective mucociliary clearance (MCC), lack of bacteria internalisation by the epithelial cells, as illustrated in fig. 1.1.5. (6) Nevertheless, the underlying molecular defects of CF patients are still the object of many studies and many hypothesis. (6)

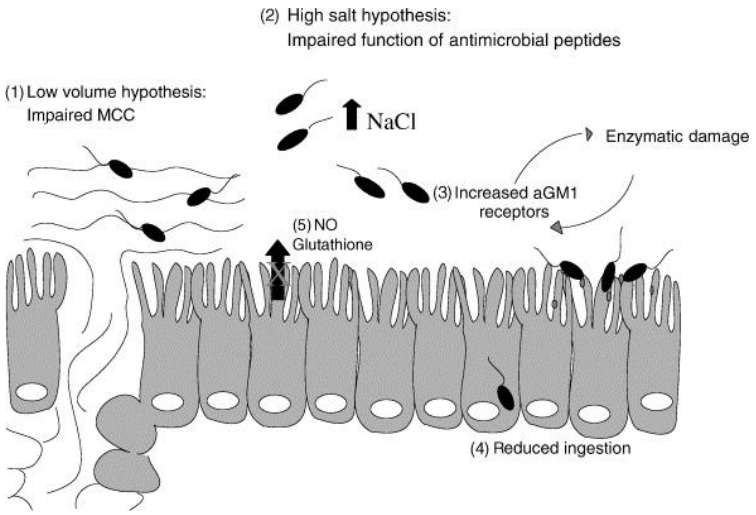


Fig.1.1.4 Hypothesis for P. aeruginosa predilection of the Cystic Fibrosis airways. *Abnormal airway surface liquid composition may lead to 1) impaired mucociliary clearance; 2) defective anti-microbial peptides. 3)increased bacterial receptors may also facilitate bacteria proliferation and 4) reduced ingestion of the microorganisms by epithelial cells. Davies JC. Pseudomonas aeruginosa in cystic fibrosis: pathogenesis and persistence. Paediatr Respir Rev. 2002 Jun 1;3(2):128–34.*

Once the bacteria have gained a foothold in the CF airways, they are able to survive and evade host defences and antimicrobial treatments, thanks to many defence mechanisms; some of these include exoproducts and antibiotic proteins secretion, phenotypic changes and formation of a thick biofilm, which shields the bacteria from the outside environment. (6)

A clear example of immune evasion is given by the downregulation of flagella by *P. aeruginosa*: once surface attachment has been achieved by the bacteria, flagella appendages are lost, making the bacteria less recognisable by the host immune system. (7)

1.1.5 Antibiotic resistance

P. aeruginosa biofilms are extremely hard to treat, particularly due to the existence of multidrug resistant strains and their wide range of intrinsic and acquired defence mechanisms. (3) Its resistance to some of the main antibiotic classes, such as aminoglycosides, quinolones and β-lactams, has caused alarmism regarding the urgent need to develop new antibiotics. (7) Resistance against antibiotic treatment in *P. aeruginosa* can either be intrinsic, acquired and adaptive. (7) Intrinsic mechanisms include limited outer membrane permeability, efflux pumps excretion and antibiotic inactivating enzymes; on the other hand, acquired resistance is characterised by horizontal transfer of resistance genes and genetic mutations; finally, adaptive antibiotic resistance is a mechanism which includes biofilm-mediated resistance, accountable for most recurrent infections of *P. aeruginosa*.(7) Here, biofilms are employed by the bacteria as a diffusion barrier, limiting access to the bacterial cell.(7)

1.1.6 Biofilm-mediated resistance in *P. aeruginosa*

On top of protecting the bacteria from antibiotic penetration, which becomes slower and more difficult, the biofilm induces physiological and phenotypic changes, which make the bacteria resistant to antimicrobial treatment. (7) Microbial cells within a biofilm are in fact less sensitive to drug agents, which is not necessarily due to genetic mutations but simply the result of adaptive mechanisms.(8) This is shown by a study where a strain of *Klebsiella pneumoniae* defective in β-lactamase production was employed: when grown in membrane-supported biofilms, it had higher minimum inhibitory concentration to ampicillin and ciprofloxacin antibiotics, as compared to when grown in suspension culture. (8) Biofilms also lead to changes

in the bacteria microenvironment, making it more acidic and suitable for bacterial growth and less suitable for antimicrobial agents. (9)Furthermore, antibiotic molecules can be sequestered by periplasmic glucans secreted in the biofilm matrix upon genetic regulation, and therefore are unable to reach the site of action.(7)

Finally, 1% of biofilms are characterised by persister cells, slow growing and metabolically inactive cells, tolerant to very high concentrations of antibiotics.(10) These are characterised by a state of dormancy, which allows them to remain viable while most of other cells are killed by antibiotics; once the antibiotics are removed, persister cells resume their growth and repopulate the biofilm. (10)

1.1.7 Biofilm diagnostic tools

Diagnosis of biofilm remains challenging due to the lack of biofilm-specific biomarkers and, most of all, the lack of a standardised protocol in clinical practice. (11) This is also due to the fact that, often, symptoms of the patients are vague and nonspecific. (11) A further problem is that identification of biofilms on medical devices, well proved to worsen patients' clinical conditions, are not consistently investigated, despite the available methods to detect them. (11)

Some of the detection methods allow biofilm structure visualisation and include Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). However, these methodologies are performed in vitro and entail analysis at a different place from where the biofilm had formed, therefore they lack accuracy. Multiphoton laser scanning microscopy could overcome this problem by detecting the biofilm in situ, along with other types of microscopy (e.g. atomic force microscopy) but are very expensive.(12) Polymerase chain reaction (PCR) can also be exploited for genetic identification of biofilm by looking at microbial colonisation around inflammatory cells and looking for biofilm-specific genes (13) Fluorescence in situ hybridization has also proven to be helpful in

biofilm detection, by employment of short fluorescence-labelled oligonucleotides which target ribosomal RNA of the microorganism. (14)

More common in vitro methods include the colorimetric Congo red agar test, the tube biofilm formation test and the microplate test. These are mainly quantitative tests and subject to chromatic evaluation, can vary among species and accordingly to the cut-off value used as reference by the clinician. Finally, some in vivo models have been employed to study biofilm but no standardisation has been provided yet and we are still far from employing them in clinical settings. Nevertheless, due to cost and lack of equipment, clinical laboratories use more conventional methods to classify pathogens, which are mostly performed on planktonic bacteria, often generating misleading results.

1.2 *P. aeruginosa* biofilm Structure

The biofilm is an intricate ensemble of bacteria, encased in a self-generated matrix composed of extracellular polymeric substances (EPS); it is one of the most crucial strategies for the survival of species during abrupt changes of living conditions (e.g. temperature variation, nutrient availability etc). (15) The biofilm structure allows bacteria to escape host immune responses and resist antimicrobial treatments making them up to 1000 times more resistant to antimicrobials than their planktonic equivalents. (15)

P. aeruginosa is well-known to form resistant biofilms, making it a superb model to study biofilm development. (15) Particularly, a robust biofilm is a critical weapon for *P. aeruginosa* to survive and prevail in the polymicrobial environment of the CF lung. (15)

1.2.1 The matrix

The matrix of the biofilm in *P. aeruginosa* mainly comprises polysaccharides, extracellular DNA (eDNA), proteins and lipids, as shown in fig. 1.2.1. (15) Responsible for more than 90% of the biofilm biomass, it provides a scaffold for adhesion to biotic and abiotic surfaces and refuge for

the enclosed bacteria during harsh environmental conditions.(15) It also provides nutrients, enzymes and cytosolic proteins for the biofilm community and facilitates cell-to-cell communication.(15) Finally, exopolysaccharides, such as Psl, Pel and alginate, are involved in surface attachment and give stability to the biofilm architecture. (15)

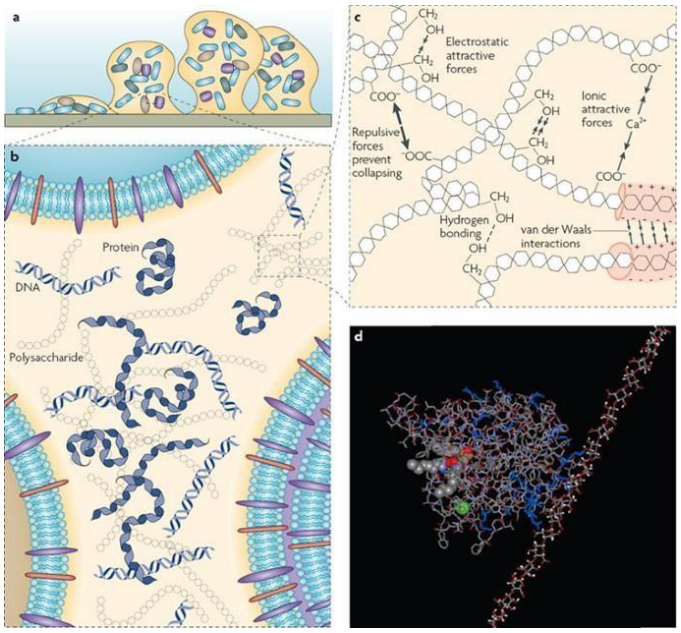


Fig 1.2.1. Biofilm composition schematic. *A) phases of biofilm growth; B) major components of biofilm such as EPS, eDNA, proteins and polisaccharides C) chemical interactions between the various saccharides D) Pymol model of a biofilm. S. Jachlewski, 2013, uisburg-Essen Publications online*

1.2.2 Types of carbohydrates involved in Biofilm

Psl: mannose-rich carbohydrate, necessary for adhesion and cell-to-cell interactions; it helps maintaining structural stability and function as signalling molecule to promote the production of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) whose level, if elevated, results in thicker and more robust biofilms; it shields biofilm bacteria from antimicrobials and neutrophil phagocytosis.(16)

Pel: polysaccharide polymer of partially deacetylated *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine; important for surface attachment and maintenance of biofilm integrity. It promotes the tolerance to aminoglycoside antibiotics.(16) The importance of Pel in biofilm

formation varies among different strains: Psl is the main exopolysaccharide produced by the common laboratory strain *P. aeruginosa* PAO1, while Pel is the most predominant exopolysaccharide in another commonly used laboratory *P. aeruginosa* strain, PA14, which is incapable of Psl production(16).

Alginate: negatively charged acetylated polymer, it comprises a mannuronic acid and guluronic acid residues.(16) It is necessary for biofilm maturation, protection from phagocytosis and opsonization and to decrease dispersal of antibiotics through the biofilm.(16) The viscoelastic properties of biofilms is influenced by the proportion between mannuronic acid and guluronic acid, which can lead to impairment of cough clearance in the lung of CF patients infected with *P. aeruginosa.* (15)

1.2.3 Other components of Biofilm: eDNA and secondary metabolites

Cell lysis, caused by environmental stress such as the antimicrobial treatment, leads to release of DNA into the environment; this extracellular DNA (eDNA) is one of the pivotal constituents of biofilms.(15) The released eDNA, along with cytosolic proteins and RNA are subsequently encapsulated into membrane vesicles (MVs) which are formed via membrane fragments originating from the lysed cells. eDNA is involved in various processes: it constitutes a source of nutrient for bacteria in the biofilm and supports cellular organization by binding to other biopolymers (exopolysaccharides and proteins) therefore stabilizing the microcolonies biofilm architecture and promoting aggregation and surface attachment.(15) Moreover, it acts as a cation chelator that interacts with divalent cations (Mg^{2+} and Ca^{2+}) on the outer membrane and subsequently activates the type VI secretion system which disseminates virulence factors within the host.(15) The deposition of eDNA causes the biofilm environment and infection sites to become acidic, limiting the penetration of antimicrobial agents (such as the antibiotics polymyxins and aminoglycosides) and also influences the inflammatory process activated by neutrophil. (15) In *P. aeruginosa*, eDNA release is regulated via the pseudomonas quinolone

signal (*PQS*) dependent quorum sensing (QS) and is primarily located in the stalks at the borders between micro-colony caps and stalks.(15) Finally, biofilm integrity is enhanced by secondary metabolites such as phenazines which intercalate with eDNA. An example is given by Pyocyanin, which contribute to DNA release through the formation of reactive oxygen species such as hydrogen peroxide, by damaging the cell membrane. (15)

1.2.4 Biofilm stages

Various stages of development lead to bacterial biofilm formation. (15) Particularly, six stages can be described, as summarised in fig. 1.2.4:

Stage I: the bacterial cells adhere to a surface via support of cell appendages such as flagella and type IV pili. This adherence is reversible; (15)

Stage II: characterised by the switch from reversible to irreversible attachment of the bacterial cells; (15)

Stage III: Progressive propagation of attached bacteria into a more structured architecture, termed microcolonies; (15)

Stage IV: These microcolonies develop further into extensive three-dimensional mushroom-like structures, a hallmark of biofilm maturation; (15)

Stage V: In the centre of the microcolony, matrix cavity is disrupted through cell autolysis for the liberation of dispersed cells; (15)

Stage VI: the transition from sessile to planktonic growth mode for seeding of uncolonized spaces which allows the biofilm cycle to repeat; (15)

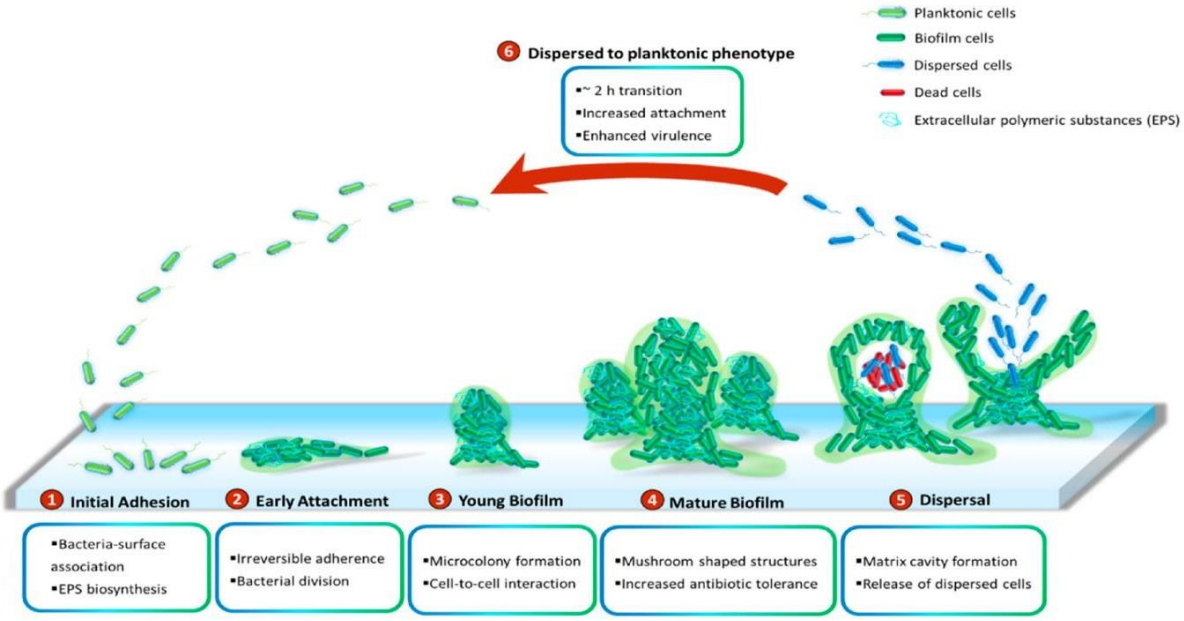


Fig. 1.2.4 Cycle of P. aeruginosa biofilm development. *Six stages can be described, leading to biofilm development: 1) initial adhesion, characterised by weak association between the bacteria and the surface and when EPS synthesis starts 2) Early attachment, when the bacteria starts dividing and increase in number 3) Young biofilm, defined by microcolony formation and cell-to-cell interactions 4) Mature biofilm, characterised by mushroom shaped structures and antibiotic resistance 5) Dispersal of cells 6) Dispersal to planktonic phenotype* Minh Tam Tran Thi, 2020, *Cycle of P. aeruginosa biofilm development.* Int. J. Mol. Sci.

1.2.5 Mushroom-like structures

The development of mushroom-like structures, typical of *P. aeruginosa* biofilm and characteristic of stage IV (fig.1.2.4), is dependent upon the available carbon and energy source. (15) The mushroom-like structure consists of stalks on which caps form.(15) Cap production requires a form of surface movement called twitching motility.(15) Apparently, cells in the stalk do not move and a subpopulation of cells use twitching motility to migrate up the stalk and form a cap. Quorum sensing (see chapter 1.3) influences the ability of *P. aeruginosa* to develop stalks, so that quorum-sensing mutants form a thin, rather uniform layer on surfaces. (15)

1.3 Mechanisms of biofilm regulation in *P. aeruginosa*

The formation of biofilm is induced and regulated by numerous genes and environmental factors, of which three are most important: 1) Quorum Sensing (QS), which controls about 10% genes in *P. aeruginosa*, 2) the bis-(3'-5')-cyclic diguanosine monophosphate (c-di-GMP) signalling network, which determines whether bacteria adopt either planktonic or biofilm phenotype, 3) small RNAs (sRNAs) although their role in biofilm is yet to be clear. (17)

1.3.1 Quorum sensing (QS)

In bacteria, QS is the regulation of gene expression in response to variations of cell-population density. *P. aeruginosa* has three QS systems: the *las*, *rhl*, *PQS* systems. (17)

The *las* system consists of LasR, a transcriptional activator, the enzyme-coding gene *lasI*, responsible for the synthesis of the autoinducer N-(3-oxododecanoyl) homoserine lactone (PAI-1) and the operon made of the genes *lasA* and *lasB*. Compared to wild type biofilm, the biofilm of *lasI* mutant appears flat, undifferentiated, and quickly dispersible, confirming the role of this quorum sensing system in biofilm formation and maturation. It has been reported that the QS regulator LasR can bind to the promoter region of the *psl* operon, suggesting that QS can regulate *psl* expression. (17)

The *rhl* system is characterised by three enzyme-coding genes *rhlA*, *rhlB and rhlC* and two regulatory genes, *rhlR* and *rhlI*. *rhlA* and *B* are organised in an operon and their transcription is induced by RhlR, a transcription factor that responds to the RhlI-generated signal, N-butanoyl-HSL(C4-HSL).(17,18) Transcription of the *pel* operon is reduced in *rhlI* mutant, confirming role of the *rhl* system in *P. aeruginosa* biofilm formation by enhancing Pel polysaccharide

biosynthesis. (17,18) Moreover, the *rhl* QS system has been shown to regulate expression of the cytotoxic virulence factors galactophilic lectins LecA and LecB, which contribute to biofilm development in *P. aeruginosa*, as shown in *lecA* and *lecB* mutants, which form thin biofilms as compared to the wild type bacteria. (18)

While the above systems exist in many bacteria, the *PQS* system, which stands for *Pseudomonas* Quinolone Signal, is only found in *Pseudomonas* strains.(19) The PQS synthesis cluster is made up of *pqsABCDE, phnAB*, and *pqsH*. The biofilm formed by *pqsA* mutants contains less eDNA than biofilm formed by the wild type, showing its involvement in eDNA release during biofilm development.(19) Moreover, research has shown a link between the PQS system and the production of other biofilm components, such as glycolipids, virulence factors and membrane vesicles. (19)

1.3.1.1 QS molecular signaling

The *las* and *rhl* are N-acylated homoserine lactone (AHL)-based QS systems, shown in fig. 1.3.1.4 in brown and yellow colour respectively: the first is controlled via the autoinducer N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) while the latter is controlled via an N-butyryl-L-homoserine lactone (BHL). (19) LasR and RhlR (the QS receptors for the *las* and *rhl* systems, respectively) homodimerize after binding their signal molecules, which allow them to connect to conserved *las-rhl* boxes in the promoters of target genes, thus prompting their transcription. On the other hand, the quinolone-based QS system, shown in fig. 1.3.1.4 in green colour, acts mainly via 2-heptyl-3-hydroxy-4-quinolone (HHQ).(19) Overall, these signalling systems create a global regulatory network and are believed to regulate the expression of up to 12% of the *P. aeruginosa* genome, as represented in the figure underneath. (19)

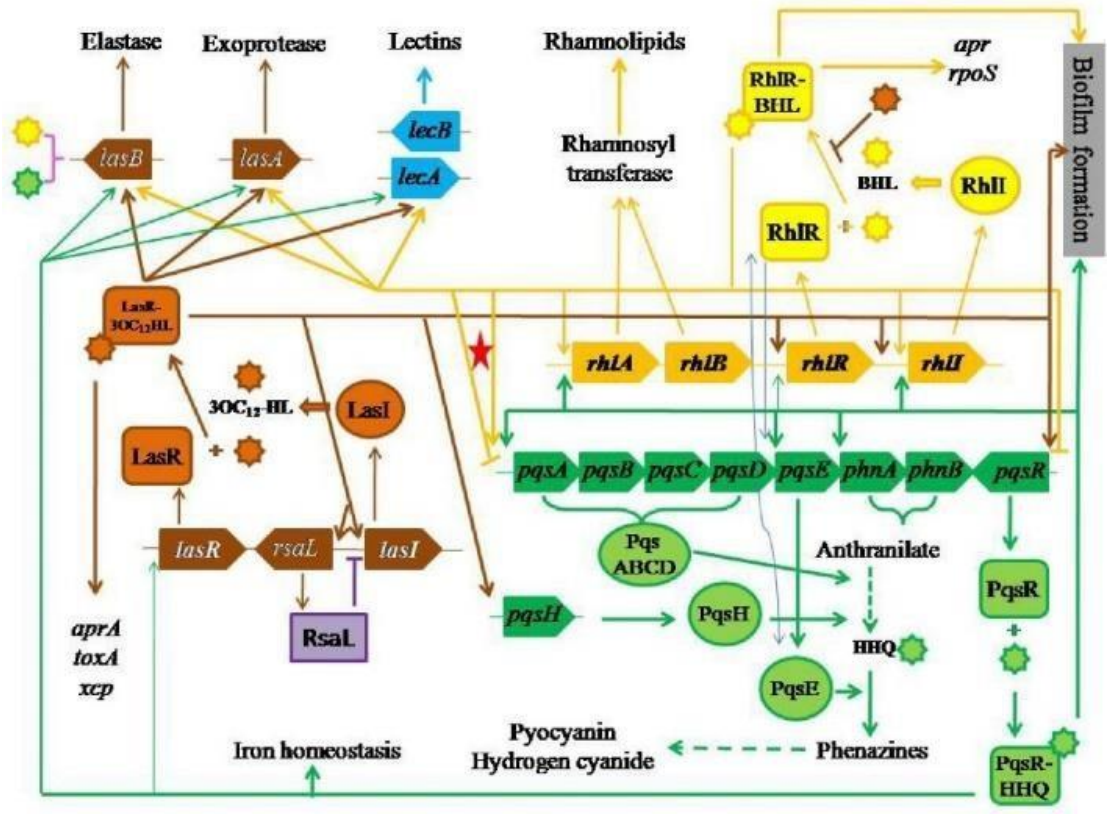


Fig. 1.3.1.1 Quorum sensing systems in P. aeruginosa. *The three main systems are shown: las system in brown, is mainly responsible for production of Elastase, Exoprotease and lectins proteins, while the rhl system in yellow provides for Rhamnolipid synthesis. Finally the PQS system in green is involved in Iron homeostasis and pyocyanin production. The three pathways are interconnected, through the action of homoserine lactone and quinolone quorum sensing molecules. S. Yan, G. Wu, 2019, Three QS systems with their effects and regulatory pathways, Frontiers in Microbiology*

1.3.1.2 QS interfering strategies

QS interference has been recently studied as a possible strategy to control bacteria, hindering biofilm formation and increasing their susceptibility to antimicrobial drugs such as antibiotics. (20) Some of these strategies include the enzymatic degradation of QS molecules and is referred to as Quorum Quenching (QQ); other mechanisms of bacterial communication disruption include QS inhibition via inhibitor molecules, autoinducers (AIs) interference by small molecules known as QS inhibitors, AIs scavenging by quorum quenching antibodies. (20) Such novel approaches seem very promising, particularly in the worrying context of antibiotic resistance, where a new approach is urgently required to treat multi-drug resistant infections.(20)

1.3.2 Other regulatory mechanisms of biofilm

An indirect link between biofilm formation and QS has also been reported, through the control of swarming and twitching motilities, as well as rhamnolipids and lectins production.(21)

The swarming motility is a form of organized surface translocation, dependent on broad flagellation and cell-to-cell contact, as shown in fig. 1.3.2.1 A; regulated by the *rhl* system, swarming motility is implicated in early stages of *P. aeruginosa* biofilm establishment.(21) On the other hand, twitching motility is a flagella-independent form of bacterial translocation, which occurs by successive extension and retraction of polar type IV pili, as shown in fig. 1.3.2.1 B. Known to be regulated by the *rhl* system, twitching motilities are important for the aggregation of a monolayer of *P. aeruginosa* cells into microcolonies. (21)

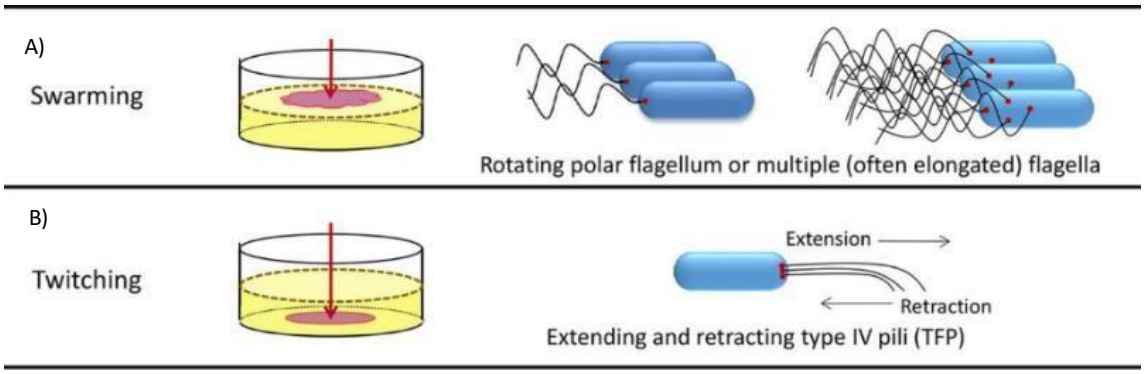


Fig.1.3.2. Swarming and twitching motility. *Diagram showing a) swarming motility in relying on flagella rotation and b) twitching motility, relying on type IV pili. Anne E. Mattingly, 2018, Bacterial surface motility modes, ASM Journals, Journal of Bacteriology*

1.3.2.1 Rhamnolipids

Rhamnolipids are a class of glycolipid produced by *P. aeruginosa*, also known as bacterial surfactants.(22) They have a glycosyl head group and an alkanoic acid fatty acid tail. Rhamnolipids are found in the sputa of cystic fibrosis patients and can inactivate tracheal cilia

of mammalian cells, indicating that they are virulence factors. (21) On top of their biosurfactant and virulence factor roles, rhamnolipids cover many roles in *P. aeruginosa* biofilm formation: 1) they are involved in swarming motility and microcolonies formation; 2) they maintain open channel structures for distributing nutrient and oxygen and for removing waste products in mushroom-shaped structures 3) prevent bacterial colonization by disrupting cell-to-cell and cell-to-surface interactions; 4) they facilitate three-dimensional mushroom-shaped structures formation; 5) they facilitate cell dispersion from the biofilm, and help bacteria to utilize long-chain fatty acids as sources of carbon.(22) As a direct consequence, biofilms produced in the absence of rhamnolipids are much less heterogeneous.(22)

1.3.2.2 Genetic regulation of rhamnolipid production

Rhamnolipid production is under the *rhl* system control, a schematic of which is shown in fig. 1.3.2.3.(23) Data indicated that the *RhlA* mutant was able to form the channels surrounding the microcolonies but was unable to maintain them.(23) *rhlA* and *rhlB* encode the two subunits of rhamnosyltransferase I, while *rhlC* encodes the rhamnosyltranferase II that facilitate the ligation of mono-rhamnolipid into di-rhamnolipids.(23) During biofilm development, expression of the *rhlAB* operon is observed in microcolonies greater than 20 µm in depth and the synthesis of rhamnolipid in *P. aeruginosa* occurs at its late-exponential and stationary phases. (23)(24)Very interestingly, data showed that a *P. aeruginosa* strain unable to produce any detectable rhamnolipids, due to an inactivating mutation in the single QS-controlled *rhlA* gene, did not induce necrosis of PMNs *in vitro* and exhibited increased clearance compared with its wild-type counterpart *in vivo*.(18) In fact, Rhamnolipids act as heat-stable extracellular hemolysins and are known to lyse polymorphonuclear leukocytes and monocyte-derived macrophages, resulting in necrotic cell death. (18)

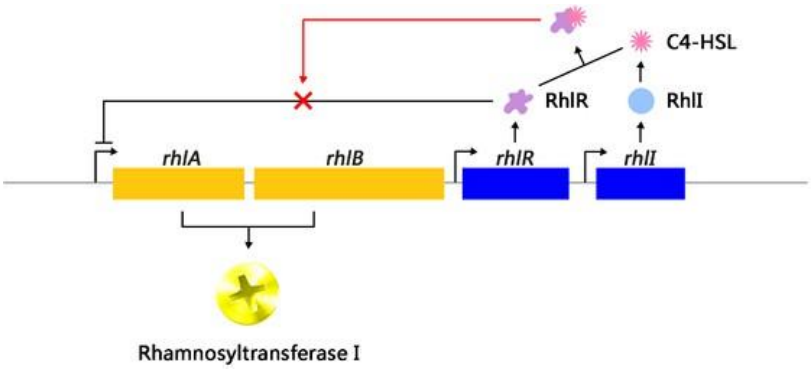


Fig.1.3.2.2 Regulation of rhl QS system. *rhlI encodes for acyl-homoserine-lactone synthase, which catalize the synthesis of C4-HSL, whose action on the transcription factor rhlR is necessary for activation of the rhlAB operon. Tianyi-Huang, 2014, Regulation model of rhlABRI, Registry of Standard biological parts*

1.4 *C. elegans* as a model organism

The organism *C. elegans* is a well-established model organism for the study of many human diseases, used in a wealth of diverse areas of biology; it is also used to look at host-parasite interactions, as well as for high throughput drug screening. (25)

The tiny nematode is found worldwide and can be easily isolated from soil and rotting vegetable matter; 1 millimetre long, it is easily handled in the laboratory setting, where it is cultivated on petri dishes containing lawns of *E. coli* bacterium. (25) Some of its advantages include the animal rapid life cycle and its well-elucidated genome, which comprises about two-thirds of all human disease-associated genes.(25) Wild type *C. elegans* can either be males or self-fertilising hermaphrodites, the second constituting a big advantage for research studies, since one animal can give rise to an entire population of about 300 self-progeny (or about 1000 offspring, if mated with males); when kept at temperatures ranging 20°-25°C, it takes them about 3 days to grow from egg to egg-laying adults, facilitating rapid experiments and collection of data. (26)

Moreover, thanks to the animal transparency, it is possible to observe its internal organs, namely the digestive system, nervous system and reproductive structures, but also internal cells and subcellular details; this can be easily achieved by using dissect microscopes, allowing up to 100X

magnification, or confocal microscopes, allowing up to 1000X resolution.(26) Also, fluorescent tags can be adopted to enhance visualisation of compartments of interest. (26)

1.4.1 *C. elegans* life cycle

At 20°C *C. elegans* embryogenesis takes about 16 hours: after fertilisation, an eggshell forms and develops into an embryo until it hatches and develops through four larval stages (L1-L4). (26)The first larval stage L1 lasts approximately 16 hours, while the next three larval stages (L2-L4) last about 12 hours; a lethargic period characterises the end of each stage, with the formation of a new cuticle layer.(26) It takes about 12 hours before the young adults start producing eggs, for a period of 2-3 days until they have used up all their self-produced sperm.(26) Adults have a life span ranging from 12 to 18 days even though it is possible to control the rate of development by variations of temperature: lower temperatures extend lifespan as opposed to higher temperatures, which shorten *C. elegans* lifespan. (26)

In cases of food depletion, L2 larvae moult into an alternative L3 larval stage called “dauer” larva. (26) This is characterised by a cuticle which surrounds the entire animal and occlude its mouth, therefore preventing food ingestion and further development.(26) The dauer cuticle is extremely resistant, rendering larvae less susceptible to environmental stresses and chemicals and able to survive for many months, until food is available so it reverts to its normal development. (26)

1.4.2 *C. elegans* gut anatomy

The intestine in *C. elegans* carries out many functions, such as food digestion, nutrients absorption, macromolecule storage and innate immunity initiation. (27) This organ comprises 20 large epithelial cells, symmetrically positioned around the lumen, forming a long tube, as shown in pink colour in fig.1.4.2 underneath. (27) The organ is attached to the pharynx through the pharyngeal intestinal valve and also to the anus, through the rectal valve.(27) In adult animals, a deflection allows the prolongation of the gonads and only one muscle, the stomatointestinal

muscle, innervates the posterior end of it.(27) The pharynx is connected to the buccal cavity, regulated by the nervous system which integrates signals related to the animal nutritional status and external signals related to the absence/presence of food, therefore increasing/decreasing the rate of pharynx pumps accordingly.(27)

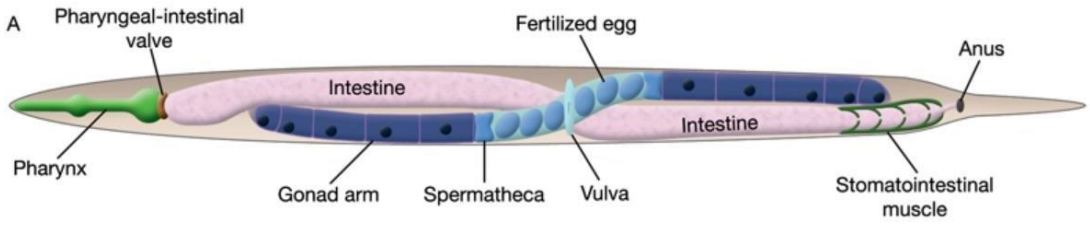


Fig. 1.4.2 C. elegans intestine anatomy. *Ventral view of C. elegans showing the pharynx and intestine, positioned on the left side, in the portion anterior to the vulva, and on the right side of the animal, in its part posterior to the vulva. Wood at al. 1996, Maternal effect of low temperature on handedness determination in* C. elegans *embryos. Dev. Genet.*

1.4.3 *C. elegans* as a model for biofilm studies

C. elegans offers the opportunity to study host-microbes interactions, since a wide variety of bacterial pathogens and fungi can infect, kill or cause nonlethal disease in the nematode.(28) Some of these include human pathogens such as gram negative bacteria of the biofilm-forming genera Pseudomonas, making it a good candidate model organism to study pathogenic biofilms. (28) Microbial pathogens usually colonize the intestine of *C. elegans*, shortening its lifespan and producing disease-associated phenotypes. (28)Other mechanisms employed by pathogenic microbes to injure *C. elegans* include production of toxins, virulence factors, hindering of the worms development, cuticle damage etc. (28)

Therefore, *C. elegans* could be employed to study bacterial biofilms, upon biofilm colonisation of the worm intestine and by comparison to biofilm-incompetent bacteria colonisation. Some of the available techniques to visualise biofilm infection in the nematode include fluorescent confocal microscopy of intestinal cells to visualise/measure bacterial fluorescence. One of the downsides of such techniques is the accumulation of material in aging *C. elegans* intestine, which auto

fluoresces under different excitation wavelengths. (29)Autofluorescence, also caused by the presence of lysosome-related auto fluorescent organelles, may hinder bacterial visualisation by mistaking such fluorescence for bacterial biofilm, such as shown in fig.1.4.3. (29)

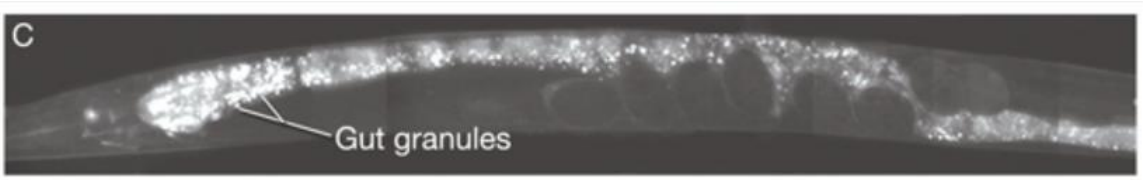


Fig. 1.4.3 Autofluorescence of C. elegans intestine. *400X magnification epifluorescent image showing auto fluorescent granules in C. elegans gut. Worm Atlas, alimentary system intestine.*

Nevertheless, a wide range of assays could also be employed in the investigations of biofilm effect onto *C. elegans* lifespan and health profile, such as survival assays, use of automated high-throughput imaging platforms to track movement and host physiology observations. Total mRNA analysis could also be employed to identify up/downregulation of host genes in response to biofilm formation, biofilm-mediated increment in oxidative stress resistance and lifespan extension. (30) Moreover, toxicity assays could be employed to assess developmental time, brood size of the worms, feeding rate, fertility, etc. (31) Finally, dissection of adult *C. elegans* intestines to isolate intestine tissue could be performed to assess the presence of biofilm components, such as by DAPI staining of viable and dead bacterial cells, CTC staining to show metabolic activity of viable cells and Alexa Fluor488 to stain Alginate. (32)

1.4.4 *glp-1* worms

glp-1 is a gene involved in the control of germ cell proliferation, during postembryonic development of *C. elegans,* as shown in fig.7. (33) It encodes for a member of the NOTCH family of receptors GLP-1 protein, mediating mitosis/meiosis and maintenance of germline stem cells (GSC) of *C. elegans* during larval development (fig.1.4.4 A, B, C).

glp-1(e2141) mutant worms are a temperature sensitive strain which, when exposed to the restrictive temperature of 25°C, fails to develop a germ-line (fig.1.4.4, E, F) and therefore are sterile. (34) This is due to the animal bearing a temperature-sensitive reduction of function *glp-1* mutation.(34) Sterile worms are conveniently used in the laboratory setting of *C. elegans* studies to avoid progeny contamination while performing survival assays.

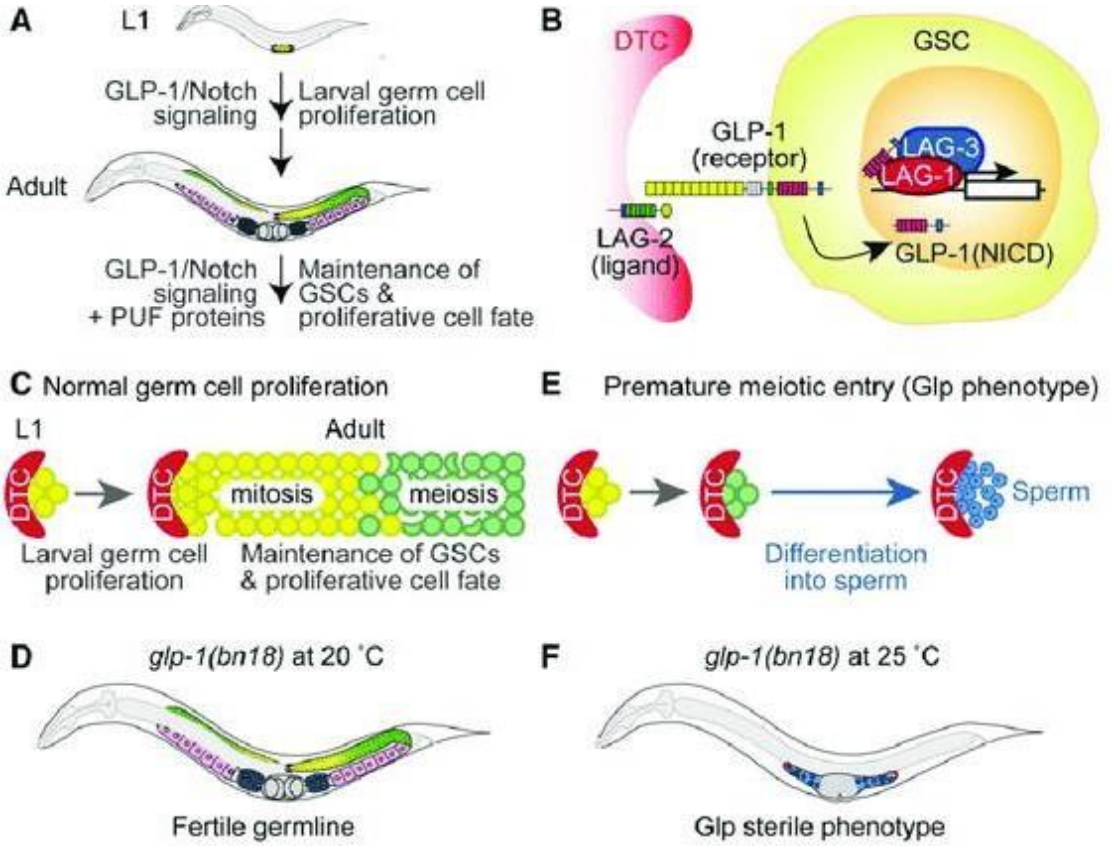


Fig. 1.4.4 C. elegans germline and GLP-1/Notch signaling pathway. *(A) GLP-1/Notch signaling pathway is required for germ cell proliferation during larval development and for GSCs maintenance during adulthood. (B) Upon GLP-1 activation, the GLP-1/Notch intracellular domain (NICD), LAG-1, and LAG-3 form a ternary complex in the nucleus and activate the transcription of target genes. (C and D) Schematics of normal germ cell proliferation and the germline phenotype of glp-1(bn18) mutant at 20 °C. In a normal germline, GLP-1/Notch signaling promotes GSC maintenance and germ cell proliferation. Once germ cells (yellow) move proximally, they enter meiosis (green). (E and F) Schematic of premature meiotic entry (also called Glp) phenotype and the germline phenotype of glp-1(bn18) mutant at 25 °C. Yoon D. Cha D. Al)ili M. et al., 2018, Subunits of the DNA polymerase alpha-primase complex promote Notch-mediated proliferation with discrete and shared functions in C. elegans germline, FEBS Journal*

1.5 Concluding remarks

Bacterial biofilms have been recognised as the cause of many chronic infections and a big threat to hospitalised and immunocompromised people. Current knowledge indicates that different mechanisms contribute to bacterial biofilms' development and pathogenesis of disease, governed by a tight regulation at genetic level. Knowledge of such mechanisms is extremely important to develop new effective treatments for biofilm infections, which, at the moment, are still untreatable. Antibiotic resistance and adaptive stress response are in fact worrying features of biofilms, which hinder antimicrobial treatments. Moreover, biofilms are extremely challenging to diagnose in clinical settings, due to lack of biofilm-specific biomarkers and lack of in vivo diagnostic tools with standardized protocols.

Genetic regulation in the biofilm-former *P. aeruginosa* is of extreme importance to form a functioning and virulent biofilm. Particularly, the QS system has been proved to play a major role in it, with the three *P. aeruginosa* QS systems, the *las, rhl and PQS* systems, being each involved in the production of essential elements for the biofilm, such as exopolysaccharides, rhamnolipids and eDNA respectively. For this reason, *las*-mutants biofilm appear flat, undifferentiated, and quickly dispersible due to lacking Psl, a mannose-rich carbohydrate, necessary for adhesion and structural stability of the biofilm. On the other hand, *rhl*-mutants form a less heterogeneous biofilm, due to lack of stable mushroom-shaped structures, typical of the late-stage *P. aeruginosa* biofilm. The *rhl* system is in fact involved in rhamnolipid production, glycoproteins which facilitate three-dimensional mushroom-shaped structures formation, by maintaining channels structures, essential for distributing nutrient, oxygen and for waste products removal. Finally, the biofilm formed by *PQS* mutants contains less eDNA, involved in various processes, such as making the biofilm environment and infection sites acidic, limiting the penetration of antimicrobial agents and acting as a nutrient source for the bacteria.

By better understanding QS regulatory mechanisms in bacteria could be a valuable resource to come up with novel approaches to defeat multi-drug resistant infections but also to come up with new biofilm diagnostic tools.

1.5.1 Project outline

This project aims to visualise biofilm into a novel whole organism biofilm model, using *C. elegans* and a vector with biofilm-specific fluorescence, which exploits Quorum Sensing genetic regulation. The first part of this projects entails the use of vector miniCTX1 to clone a construct which allows visualization of GFP green fluorescence and mCherry red fluorescence in the same cell at the same time. The green fluorescence will be constitutively expressed, while mCherry would only be expressed when the cloned promoter of biofilm-related genes, such as *rhlA, rhlB, rhlI, pqsA and pqsB* will also be expressed. This design will allow to tell when the bacteria is expressing the above mentioned genes and therefore forming a biofilm, by the visualisation of a new shade of fluorescence, as a result of the mixing of the green and red fluorescence. On the contrary, green fluorescence will mark the presence of planktonic cells. The genes *rhlA, rhlB, rhlI, pqsA and pqsB* have been chosen for their implication into the biofilm forming process, particularly related to rhamnolipid and eDNA release, two essential components for the formation of a virulent biofilm.
C. elegans has been chosen as a model organism, thanks to the many advantages offered by the nematode, on top of which its transparency, which allows fluorescence monitoring using epifluorescence and confocal microscopy.

The overall goal of this project is to make an easy assay which could be available to industry and academic researchers: this is done by using *glp-1* worms, which are sterile at 25°C, therefore are easily handled and convenient for infection assays without the need of clearing the plate from progeny each day. Worms are synchronized by bleaching them, then fed and grown onto *E. coli* OP50 bacterial lawns until larval (L4) stage, which is when they get transferred into biofilm-

competent bacteria which carry a fluorescent reporter. Finally, worms are monitored throughout their adulthood to see the biofilm developing inside them.

The second part of this project investigates the possibility of employing already existing fluorescent biofilm markers available in the research community to study biofilm in *C. elegans*. A range of in vitro and in vivo assays will be employed to look at biofilm formation in different *P. aeruginosa* transgenic strains, namely the microtiter biofilm assay, fluorescence based assays, *C. elegans* survival assay, *C. elegans* healthspan readouts and in vivo imaging with epifluorescent microscopy.

At the moment, biofilm tests are performed in cultured cells, without providing a biofilm-host interaction nor a host health readout. (11)This makes the study of biofilm difficult and inaccurate.(11) For this reason, providing a commercialised in vivo biofilm model could be extremely useful to industry and academic researchers, in order to fill a market gap.

1.5.2 Aims and objectives

This project aims to evaluate methods to monitor *P. aeruginosa* biofilms in real-time in *C. elegans*, with two main goals:

1. Develop a quick and cost-effective color-based assay to monitor *P. aeruginosa* biofilms in real-time in *C. elegans*
2. to investigate the possibility of employing already existing fluorescent biofilm markers available in the research community to study biofilm in *C. elegans*.

Therefore, the objectives of the project are:

1. isolation of miniCTX1 vector from E. coli bacterial cultures
2. gDNA extraction from *P. aeruginosa*
3. *rhlA, rhlB, rhlI, pqsA* and *pqsB* genes' promoters amplification from gDNA

4. Cloning of *rhlA, rhlB, rhlI, PqsA* and *pqsB* genes' promoters into miniCTX1 vector
5. Transformation of miniCTX1 vector into *E.coli* DH10β competent cells
6. Generate transgenic *P. aeruginosa* strains expressing miniCTX1 vector for biofilm-specific fluorescence
7. Infection of *C. elegans* intestine with *P. aeruginosa* transgenic strains
8. Measure *C. elegans* intestinal bacterial expression of fluorescence using DMR epifluorescence microscopy and compare between biofilm-competent and incompetent strains
9. measure and compare survival of *C. elegans* infected with biofilm-competent and biofilm-incompetent transgenic *P. aeruginosa* strains
10. measure health profiles of *P. aeruginosa*-infected *C. elegans* using Magnitude Biosciences' automated high-throughput imaging platforms
11. in vitro measurement of bacterial fluorescence expression using a fluorescence microplate plate reader and comparison between biofilm-competent and biofilm-incompetent transgenic *P. aeruginosa* strains
12. in vitro quantification of biofilm and comparison between biofilm-competent and biofilm-incompetent transgenic *P. aeruginosa* strains using microtiter dish biofilm assay and absorbance microtiter plate reader
13. in vitro fluorescence measurement of biofilm-competent transgenic *P. aeruginosa* strains after treatment with the biofilm inhibitor molecules 4-Nitropyridine-N-oxide
14. measure and compare survival of *C. elegans* after intestinal infection with biofilm-competent transgenic strains treated with the biofilm inhibitor molecules 4-Nitropyridine-N-oxide

CHAPTER 2: MATERIALS AND METHOD

2.1. Materials

2.1.1 *C. elegans* strains

glip-1(e2144) *III*: *C. elegans* characterised by temperature sensitive loss of *glip-1*(e2144) activity, which limits germline proliferation. Maintained at 15°C, sterile at 25°C.

2.1.2. Nematode Growth Medium (NMG).

Nematodes were grown onto Nematode Growth Medium (NGM), following a previously established protocol. (35) For 1.6L of NMG, 27g of Agar, 4g of BactoTM Peptone and 4.8g of NaCl are topped up with 1.6L of dH2O. The mixture is first autoclaved and once cooled to 55°C, 40ml of KH2PO4 (1 M) and 1.6ml of: cholesterol (12.93mM in ethanol), $MgSO4$ (1 M) and CaCl · 2H2O (1 M), are added and mixed well. 10 ml of the mixture is then poured in 6cm plates and let solidify overnight.

2.1.3 Bacterial strains

Table 2.1.3 lists the bacteria strains used in this study, and where they were sourced from.

Table 2.1.3. Bacterial strains used in the study.

Strain name	Species	Origin
PA14 wild type	*Pseudomonas sp.*	Becky Hall, University of Kent
PA14 with *ExoS* GFP reporter (plasmid pJNE05)	*Pseudomonas sp.*	Becky Hall, University of Kent
PA103 wild type	*Pseudomonas sp.*	Becky Hall, University of Kent
PA103 with *ExoS* GFP reporter (plasmid pJNE05)	*Pseudomonas sp.*	Becky Hall, University of Kent
PA01 wild type	*Pseudomonas sp.*	Gary Robinson, University of Kent
PDO-100 PAO1 Δ*RhlI*; with GFP expressed constitutively from lac promoter (plasmid pTdK-GFP)	*Pseudomonas sp.*	Gary Robinson, University of Kent
PAO-JP1 PAO1 Δ*lasI* with GFP expressed constitutively from *lac* promoter (plasmid pTdK-GFP)	*Pseudomonas sp.*	Gary Robinson, University of Kent
PAO-JP2; PAO1 Δ*rhlI* Δ*lasI* with pTdK-GFP	*Pseudomonas sp.*	Gary Robinson, University of Kent

E. coli BL21	*Escherichia coli*	Becky Hall, University of Kent
E. coli OP50	*Escherichia coli*	Marina Ezcurra, University of Kent

SM381 - *PrhlA-mNeonGreen*	*Pseudomonas sp.*	Bonnie Bassler, Princeton University
SM383 - Δ*rhlR PrhlA-mNeonGreen*	*Pseudomonas sp.*	Bonnie Bassler, Princeton University

2.1.4. Bacterial culture Media

Bacteria were grown in Luria-Bertani (LB) with the antibiotic at the required working concentration, as listed in table 2.1.4. To make 1L, 25g of LB granules were dissolved in 1L of dH2O and autoclaved, as by standard protocol. (35) Detailed information about bacterial strains and their growth conditions is provided in table 2.1.4.

Table 2.1.4. List of Growth conditions for each bacterial strains.

Strain name	Growth condition	Antibiotic working conc.
PA14 wild type	LB only	
PA14 with pJNE05	LB + Gent	25 µg/mL
PA103 wild type	LB only	
PA103 with pJNE05	LB + Gent	200 µg/ml
PA01 wild type	LB only	
PDO-100 with pTdK-GFP	LB + CARB	200 µg/ml
PAO-JP1 with pTdK-GFP	LB + CARB + TET	Carb-200 µg/ml Tet-50 mg/mL
PAO-JP2 with pTdK-GFP	LB + CARB + TET	Carb-200 µg/ml Tet-50 mg/mL
E. coli BL21	LB only	
E. coli OP50	LB only	
Sm381 - *PrhlA-mNeonGreen*	LB only	
Sm383 - *DrhlR PrhlA-mNeonGreen*	LB only	

2.1.5 Cells and plasmid vector

E.coli DH10β competent cells and the site-specific gene integration system for Pseudomonas aeruginosa miniCTX1 vector were obtained from the Hall lab (University of Kent).

The plasmid construct was developed to integrate exogenous DNA fragments at a defined site, within the genome of *Pseudomonas aeruginosa*. (36) As shown in fig. 2.1.5, the vector contains a tetracycline selectable marker (tet), an oriT for plasmid transfer through conjugation, a modified phage CTX integrase (int) gene and a multiple cloning site (MCS) flanked by T4 transcriptional termination sequences and the phage CTX attachment site. (36) Flippase enzyme (Flp) recombinase binding sites are also flanking the MCS and T4 transcriptional termination sequences, to allow excision of undesired backbone sequences. (36)

As illustrated in fig. 2.1.5 B, an expression cassette was originated by the Hall lab and cloned into the MCS of vector miniCTX1; the construct was designed to allow visualization of both red and green fluorescence at the same time, within transfected cells, driven by a promoter of choice. Therefore, it contained a constitutively expressed GFP reporter and an mCherry reporters, cloned without the ATG start codon and lacking a ribosome binding site, to prevent expression until a promoter is cloned. The stop codon has also been modified to TGA, commonly found in Pseudomonas strains. The cassette contains restriction sites for SacI, NotI and SpeI within the promoter region, and also BamHI, EcoRI, XhoI and KpnI restriction sites, to allow removal of unwanted modules (see fig. 2.1.5 B). We wanted to use this construct to clone promoter regions of genes involved in biofilm production, such as *rhlA, rhlB, rhlI, pqsA, pqSB,* to drive red fluorescence expression, upon biofilm formation from the bacteria; therefore orange fluorescence would be generated, resulting from the mix of the constitutively expressed green fluorescence and biofilm-dependent red fluorescence, as shown by fig. 2.1.5 B.

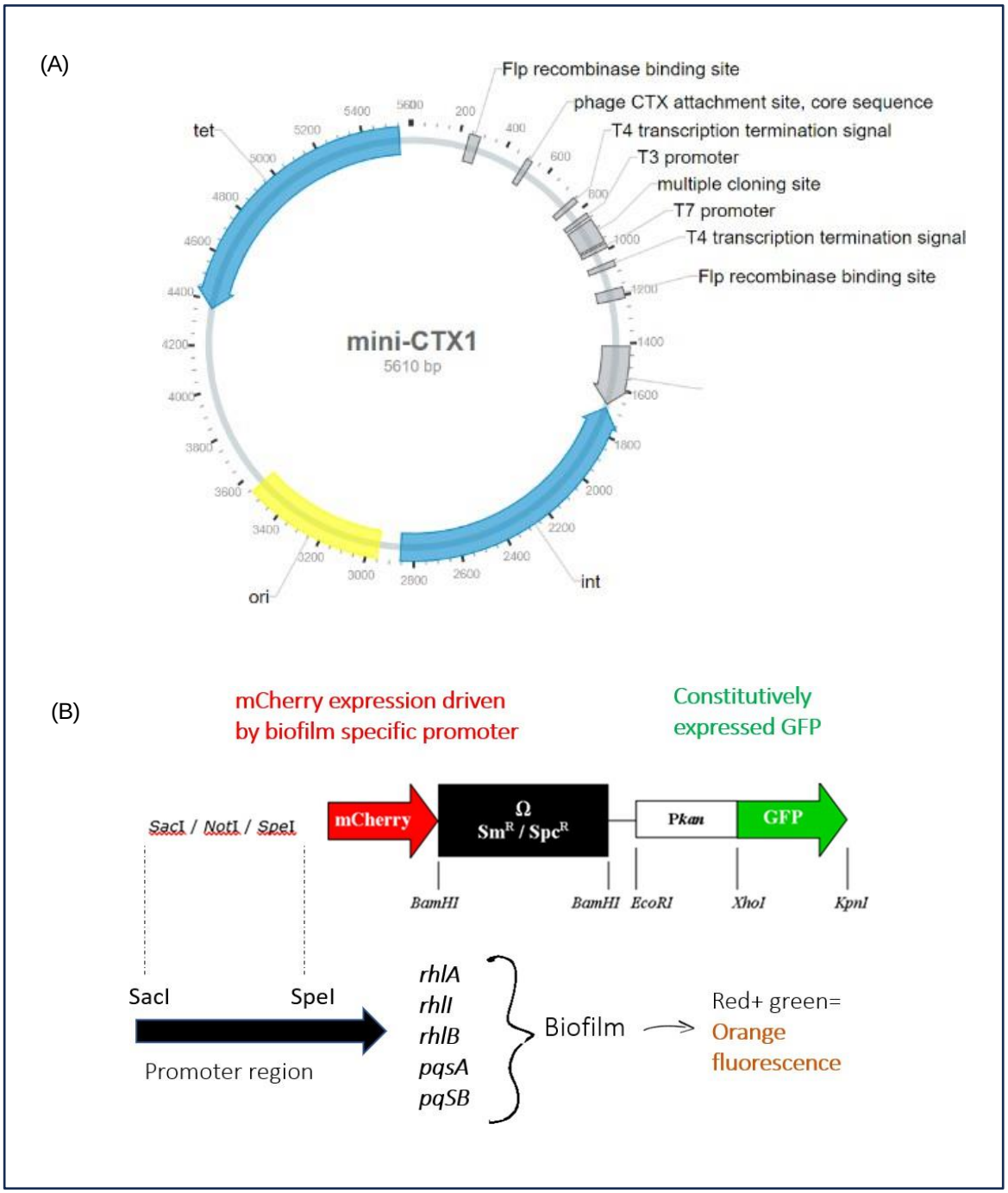


Fig. 2.1.5 Map of Vector mini CTX1 and the cloned construct. *A) Mini-CTX1 vector, characterised by 5610 basepairs length and tetracycline antibiotic resistance, also characterised by a multiple cloning site where B) the below construct had been introduced. The construct possesses an mCherry reporter and constitutively expressed GFP tag. Restriction sites are present for SacI, NotI and SpeI. B) expression cassette containing constitutively-expressed GFP and mCherry fluorescent reporters, the second driven by a biofilm-related gene's promoter. Upon biofilm expression, the generated red signal would mix with he green signal, generating orange fluorescence.*

2.1.6 Chemicals and reagents

Table 2.1.6 underneath lists all the reagents used for each technique employed during the project.

Table 2.1.6 List of reagents and Manufacturers,

Technique	Reagents
Electrophoresis	Ethidium Bromide 10mg/mL TAE (Tris-acetate-EDTA) Agarose powder, Melford 1kb Plus DNA Ladder, Invitrogen
Culture media	Miller Luria-Bertani broth, Fisher Scientific Agar, Melford
Nematode Growth Media (NGM)	Bacto™ Peptone Becton, Dickinson and Company Sodium Chloride (NaCl), Fisher Scientific Potassium Phosphate (KH2PO4), Melford Cholesterol, Sigma-Aldrich Magnesium sulphate ($MgSO4$), Melford Calcium Chloride (CaCl), Fisher Scientific
Bleach solution	10-15% sodium hypochlorite 5 M NaOH
Biofilm assay	Mueller Hinton broth, Thermo Scientific Crystal Violet Ethanol Phosphate Buffered saline (PBS) M9
Cloning	S24 plasmid BamHI, SacI, SpeI restriction enzymes Thermo Fisher Q5 High-Fidelity 2X Master Mix Primers, Sigma-Aldrich: rhlA_FWD/Rev; RhlB_Fwd/Rev; RhlI_Fwd/Rev; PqsA_Fwd/Rev; PqsB_Fwd/Rev 10X Fast Digest buffer, Thermo Fisher 10X MultiCore Buffer, Promega Acetylated BSA 1μg/μl, Thermo Fisher FastAP Thermosensitive Alkaline Phosphatase, Thermo Fisher FastAP Thermosensitive Alkaline Phosphatase 10X Reaction Buffer 2X Ligation buffer, Promega T4 ligase, Promega *E. coli* DH10β competent cells
	Chemicals
Biofilm inhibition assay	4-Nitropyridine-N-oxide (NPO), Thermo Fisher

2.1.7 Buffers

To make 1L of 1X Phosphate buffered saline (PBS), 800ml of distilled water ddH2O was added to 8 g of 0.1M Sodium Chloride (NaCl), 0.2 grams of 0.002M Potassium Chloride (Na2HPO4), 1.44 g of 0.01M Sodium Phosphate Dibasic (NaH2PO4) and 0.245g of 0.001M Potassium Phosphate Monobasic (KH2PO4) until the volume was 1L and adjusted to pH 7.2.

50X Tris-acetate-EDTA (TAE) buffer was prepared by adding 50 mL Ethylenediamine tetraacetic acid (EDTA) (pH 8.0) to 28.6 mL Glacial Acetic Acid and 121 grams Tris Base to 1000 mL ddH2O and stored at room temperature.

To prepare 1L of M9 buffer, 3 g of Potassium Dihydrogen Phosphate (KH_2PO_4) was added to 6g of Disodium Phosphate (Na_2HPO_4), 5g of Sodium Chloride (NaCl) and 1ml of 1M Magnesium Sulphate (MgSO4) and dissolved in 1L distilled water and sterilised by autoclaving for 15 minutes.

2.1.8 Kits

Commercial kits were purchased from Qiagen to perform PCR clean-up and for culture miniprep, as shown in table 2.1.8.

Table 2.1.8 Kits used for cloning.

Technique	Kit
PCR clean-up	Qiagen Gel extraction kit QIAquick Gel extraction kit
Culture Miniprep	Qiagen Miniprep kit

2.2 Worm husbandry

C. elegans glp-1 worms were grown and maintained in 15 °C incubators, onto NMG plates (prepared as described in chapter 2.1.20) seeded with a bacterial source of food. The most standard laboratory food source is OP50 *E. coli*. (35) *E. coli* was cultured in LB medium (prepared as described in chapter 2.1.4), left to grow overnight at 37°C on a shaking incubator at 180rpm.(35)

NGM plates were seeded the following day with 250 µl of bacterial culture which was left to dry overnight, before transferring the worms onto the solidified bacterial lawn. (35)A sterilized platinum wire pick was used to pick and transfer worms from plates or to eliminate dead worms.(35) To reach sterility, worms were incubated at 25°C throughout the experiment, but maintained at permissive temperatures 15-20°C. (37)

2.2.1 Synchronization

A bleaching technique is used for synchronisation of C. elegans at larval stage L1. (38)The basis of such technique implies that the nematodes are sensitive to bleach, contrarily to the egg shell which protects embryos.(38) Therefore, after treatment with an alkaline hypochlorite solution, eggs will hatch, generating a synchronised population of worms. A bleaching solution is used, containing 10-15% sodium hypochlorite, 5 M NaOH and distilled water. (38)A NMG plate populated with gravid adults is firstly washed with 1 ml of M9, which are then transferred with a pipette into Eppendorf tubes.(38) These are then centrifuged for 2 minutes at 1500 rpm at room temperature, in order to collect the worms at the bottom as pellet, gently remove the M9 supernatant and replace it with bleach solution. (38)The pellet is dissolved in the liquid by gently tapping onto the tube or by vortexing; worms are treated with the solution for approximately 3 to a maximum of 5 minutes, to avoid overbleaching, until the nematode bodies start breaking down, aiming to not get intact corpses, otherwise eggs won't get out. (38)A dissecting microscope can be used to visualise the destruction of adult tissue.(38) Next, the supernatant bleach is quickly removed by washing the worms three times, by filling the tubes with M9, centrifugation and supernatant removal. (38)After the washes, eggs are resuspended in M9, spread onto an NGM plate seeded with E. coli OP50 and left overnight to hatch. (38)This allows synchronisation to L1 stage.

2.2.2 Survival assay

The survival assay was carried out using *glp-1 C. elegans* strain. Two models of infection were initially explored: infection from eggs and infection from L4. Once established the most suitable model, two different methods were also tested: agar-based solid culture system and LB broth-based liquid culture system, based on standard lifespan protocols(39), adjusted for glip-1 temperature-sensitive worms(37).

2.2.2.1 Infection from egg-lay on solid culture system

After worms synchronisation by bleaching (38,39), eggs were placed onto NMG agar plate seeded with *E. coli* OP50 as bacterial food, incubated at 15°C. After about 5 days, when the animals reached adulthood, 10-20 young adults were transferred onto NGM plates with pseudomonas lawns, seeded the day before with 250 µl of Pseudomonas liquid culture, as per standard procedure (35). The worms were left for about 4 hours, in order to get 50-100 eggs, before being removed with a sterile platinum wire, as described in 2.2.1. Eggs were left to hatch, incubated at 15°C. Once the end of development (L4) was reached, the plate was shifted at 25°C to obtain the worms sterile phenotype.(37) The number of live worms was regularly counted as by standard lifespan solid assay, and dead worms removed from the NGM plate with a platinum wire picker, flamed each time after having touched the NGM plate, to avoid contamination of the worm stock.(39) Worms are considered dead when not responding to a gentle touch with the sterilised platinum wire.(39) On the contrary, healthy/live status can be determined by observing avoidance from a heated platinum wire positioned close to the animal. (39)Missing worms are censored but included in later statistical analysis. (39)

2.2.2.2 Infection from L4 on solid culture system

Figure 2.2.2.2 shows a simple schematic of how this assay was performed. After worms synchronisation by bleaching, eggs were placed onto a NMG agar plate seeded with *E. coli* OP50 as bacterial food, incubated at 15°C, once the animals that hatched from the eggs reached L4 stage, they were transferred to fresh biofilm forming bacteria-seeded NGM plates and incubated at 25°C, to reach a sterile phenotype(37). The number of live worms was regularly counted and dead worms removed from the NGM plate with a platinum wire picker, flamed each time after having touched the NGM plate, to avoid contamination of the worm stock. Worms are considered dead when not responding to a gentle touch with the sterilised platinum wire.(39) On the contrary, healthy/live status can be determined by observing avoidance from a heated platinum wire positioned close to the animal. Missing worms are censored but included in later statistical analysis(39).

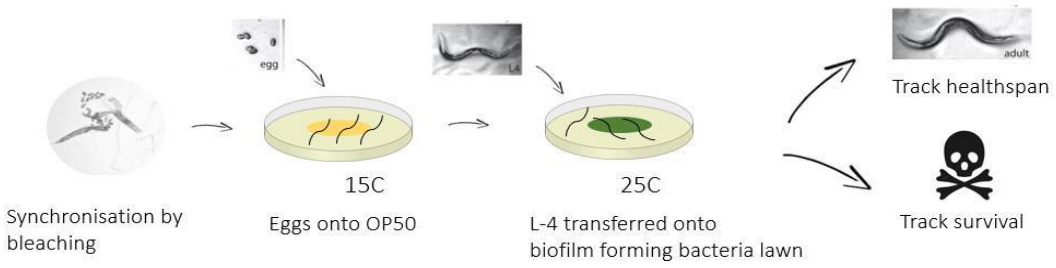


Fig. 2.2.2.2 Schematic illustrating the solid survival assay protocol. *Worms are synchronised with a bleach solution and eggs grown at 15C onto E. coli OP50 lawns until L4 larvae are picked and transferred to Pseudomonas lawns and shifted to 25C. Healthspan and survival of the worms are tracked. Created using Microsoft words.*

2.2.2.3 LB broth-based liquid culture system

As shown in fig 2.2, this experiment was conducted in a 96-well plate, with a maximum volume of 100 µL media into each well, following an already established multi-well plate infection model.(40) The media consisted of 20µL of bacterial culture and 80 µL of M9 buffer [3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilize by autoclaving.] The nematodes were initially grown onto solid media at 15°C, eggs collected from gravid adults by using bleaching(38), larvae

grown onto OP50 lawns and, once L4 larvae stage was reached, transferred into the liquid media by using a sterilized platinum wire and incubated at 25°C to induce sterility in the worms(37). Live worms were counted at two different timepoints: day 3 and day 5. To avoid drying up of the media, therefore altering the conditions and potentially affecting the results of the study, the plate was kept into a closed plastic box, kept humid with a wet towel and the most external wells of the plate were filled with 100 µL of sterile water. Worms which did not display any movement were counted as dead.

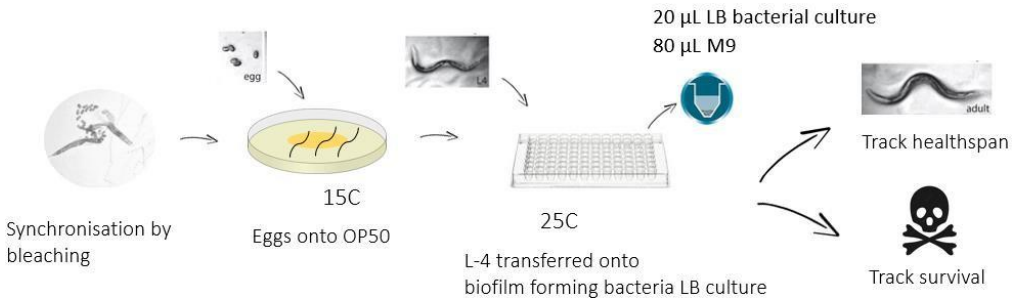


Fig. 2.2.2.3 Schematic illustrating the liquid survival assay protocol. *Worms are synchronised with a bleach solution and eggs grown at 15C onto E. coli OP50 lawns until L4 larvae are picked and transferred to a microtiter plate with LB Pseudomonas culture, shifted at 25C. Healthspan and survival of the worms are tracked. Created using Microsoft words.*

2.3 Culturing the bacterial strains

A frozen stock of each *Pseudomonas* or *E.coli* strains was made by adding the overnight culture to a cryogenic vial with 20% glycerol, mixed and vortexed and stored at -80°C.(41) After overnight static incubation at 37°C, streak plates were stored at 4°C up to one week long. *Pseudomonas* liquid cultures were set up from fresh agar plates containing antibiotic, streaked the day before from the frozen stock and incubated at 37°C static overnight, as by standard protocol.(42) Liquid cultures were set up by picking one colony of the bacteria and inoculating it in LB, using a shaking incubator and left overnight for ~16 hours at 37 °C with aeration. (42) Antibiotics were added to LB at the occurrence, see table 2.1.4. NMG plates were seeded with 250µl of the bacterial solution and left to dry and grow for 1 day before culturing *C. elegans* on it.

2.4 Cloning of *rhlA, rhlB, rhlI, pqsA* and *pqsB* promoters into mini-CTX plasmid

2.4.1 Isolation of plasmid by miniprep

Plasmid S24 was miniprepped from overnight *E. coli* bacterial cell cultures. *E. coli* strain had previously been transformed with S24 plasmid and stored in glycerol stock at -80°C. Two days before plasmid isolation, bacteria were streaked on agar plates, incubated overnight at 37°C static; the day after, cultures were grown overnight in 10 ml LB and tetracycline at 37°C on a shaker. The next day, Quiagen Miniprep kit was used on the cultures according to the manufacturer's instructions. Plasmids were eluted in 50µL EB using a spin column. DNA concentration of the plasmid was measured using a Nanodrop spectrophotometer and aliquots were stored at -20°C. Absorbance at 260/280 of the sample was also measured using a spectrophotometer, to assess DNA purity.

2.4.2 Quality check of mini-CTX plasmid

2.4.2.1 Visualisation of plasmid on 1% agarose gel

The extracted vector was gel purified using a 1% agarose gel electrophoresis. 1% agarose gel was prepared by mixing 1g of agarose powder into 100ml 1x TAE and, once cooled down, by adding ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL.1 µL of plasmid sample was added to 9 µL sterile MQ water and 2 µL 6x loading dye, then loaded onto the gel and run at 125V for 25 minutes. Bands were checked to confirm complete purification using a UV light box.

2.4.2.2 Plasmid size check by restriction digest

A restriction digestion of the plasmid with BamHI only and SacI and SpeI was carried out to check that the plasmid had the correct size. For the SacI/SpeI digest there should be only one band visible. A reaction of 20µL was set up for each combination of enzyme, using 2µL of the Thermo FastDigest 10X buffer, mixed with water, 5µL plasmid DNA and 1µL of the required enzyme, as

described in table 2.4.2.2. After 2 hours incubation at 37°C, 20µL of sample was run on a 1% agarose gel at 125V for 25 minutes.

Table 2.4.2.2 Restriction Digest reaction.

component	Volume (µL)	component	Volume (µL)
water	12 µL	water	11 µL
10X FastDigest buffer	2 µL	10X FastDigest buffer	2 µL
Plasmid DNA	5 µL	Plasmid DNA	5 µL
BamHI	1 µL	SacI	1 µL
		SpeI	1 µL

2.4.3 Genomic DNA extraction from *P. aeruginosa*

Genomic DNA (gDNA) was extracted from *P. aeruginosa* PA14 from an overnight bacterial cell culture. A single colony was inoculated into 5 ml LB and incubated overnight at 37°C on a shaker at 250rmps. DNA purification was performed using QIA-prep Spin Miniprep kit according to manufacturer's instructions.

2.4.4 Primer design

In order to clone the genes of interest (*rhlA, rhlB, rhlI, PqsA* and *pqsB*) into vector mini CTX, both the inserts and the vector had to be modified to contain appropriate restriction sites. The first insert, rhlA, was modified with the forward primer 5'GGAGAGGAGCTCAGGCCTGCGAAGTGTCCT3' and reverse primer 5'GGAGAGACTAGTTTCGCGCCGCATTTCACA3'. Insert *rhlB* was modified with the forward primer 5'GGAGAGGAGCTCTCGGCGATCGGCCATCTG3' and reverse 5' GGAGAGACTAGTGATGGCGTGCATGGGGCT3', *rhlI* was modified with the forward primer 5'GGAGAGGAGCTCTGGCGCGCGACCAGCAGA3' and reverse 5' GGAGAGACTAGTCAATTCGATCATGACCAA3', *pqsA* insert was modified with the forward primer 5'GGAGAGGAGCTCGAAGCCTGCAAATGGCAG3' and reverse primer 5' GGAGAGACTAGTCAATGTGGACATGACAGA3', while insert *pqsB* was modified with the forward primer 5'GGAGAGGAGCTCGTGCTGTTGGTGCGTGGC3' and reverse 5'

GGAGAGACTAGTCTGAATCAACATGCCCGT3'. Each forward and reverse primers included SacI and SpeI restriction sites, respectively (coloured in red). The primers have been designed to target a 500 bp region upstream of the ATG start codon of the gene of interest, including the promoter region and the first few codons, to ensure a good level of expression.

2.4.5 Inserts amplification

Genomic DNA previously isolated from *Pseudomonas aeruginosa PA14*, was used to amplify the genes of interest using a thermal cycler for Q5 PCR amplification; restriction sites were also introduced to the 5' and 3' end of each sequence. gDNA was diluted 10x in sterile MQ water and primers were diluted to a concentration of 10µM with sterile MQ water too. A 50µL reaction was set up, adding 25µL of Q5 High-Fidelity 2X Master Mix, 2.5µL of Forward and reverse primer, 1µL of PA14 gDNA and 19µL water, as shown in table 2.4.5.1. The online tool NEB temperature calculator was used to estimate the appropriate PCR conditions for each insert, as listed in table **2.4.5.1** the PCR reactions were carried out with an initial denaturation step for 30 seconds at 98°C, followed by 30-35 cycles (98°C for 10 seconds, 70°C for 20 seconds, 72°C for 30 seconds and 72°C for 30 seconds) and a final extension step at 72°C for 2 minutes. Samples were automatically kept on hold at 4°C by the thermal cycler (see tab 2.4.5.2).

Table 2.4.5.1 PCR reaction components.

Component	50 µl Reaction
Q5 High-Fidelity 2X Master Mix	25 µL
10 µM Forward Primer	2.5 µL
10 µM Reverse Primer	2.5 µL
PA14 gDNA (original stock diluted 1 in 10)	1 µL
Water	19 µL

Table 2.4.5.2. PCR reaction details. Annealing temperature was calculated using the NEB Tm calculator.

rhlA		
step	**temperature**	**time**
Initial denaturation	98 ºC	30 sec
30-35 cycles	98 ºC	10 sec
	70 ºC	20 sec
	72 ºC	30 sec
Final Extension	72 ºC	2 min
Hold	4-10 ºC	infinite
rhlB		
Initial denaturation	98 ºC	30 sec
30-35 cycles	98 ºC	10 sec
	72 ºC	20 sec
	72 ºC	30 sec
Final Extension	72 ºC	2 min
Hold	4-10 ºC	infinite
rhlI		
Initial denaturation	98 ºC	30 sec
30-35 cycles	98 ºC	10 sec
	57 ºC	20 sec
	72 ºC	30 sec
Final Extension	72 ºC	2 min
Hold	4-10 ºC	infinite
pqsA		
Initial denaturation	98 ºC	30 sec
30-35 cycles	98 ºC	10 sec
	60 ºC	20 sec
	72 ºC	30 sec
Final Extension	72 ºC	2 min
Hold	4-10 ºC	infinite
pqsB		
Initial denaturation	98 ºC	30 sec
30-35 cycles	98 ºC	10 sec
	64 ºC	20 sec
	72 ºC	30 sec
Final Extension	72 ºC	2 min
Hold	4-10 ºC	infinite

2.4.6 Gel purification and extraction of the inserts

After pcr amplification the inserts were gel purified to confirm amplification and correct size of 536bp by electrophoresis. The same procedure was followed as described in 2.4.2.1. An aliquot of 10µL of each of the PCR products was added to 2µL 6X loading dye and run for 25minutes at 125V. Bands were extracted from the agarose gel using Qiagen Gel extraction kit protocol and stored at -20°C.

2.4.7 Restriction digests of plasmid vector and inserts

After pcr amplification to amplify the inserts, both the plasmid vector and inserts were digested with restriction enzymes SacI and SpeI to create sticky ends and to make the vector compatible with the inserts. 1µg of plasmid DNA or insert was digested by setting up a 20 µL reaction with sterile MQ water and 5µL Master Mix, composed of 2µL of 10X MultiCore buffer, 2µL of Acetylated BSA and 0.5µL of Thermo Fisher FastDigest SacI and SpeI (tab 2.4.7.1). Depending on the DNA concentration of each insert, a different volume was added to the mix, as shown in tab 2.4.7.2. Each reaction was incubated at 37°C overnight. The QIAquick PCR Purification Kit was used to clean up the digests and eluted in 30µL EB buffer.

Tab 2.4.7.1 Master mix components.

Master mix 10x		
Component	**1x mix**	**10x mix**
10x MultiCore Buffer	2 µL	20 µL
Acetylated BSA 1 µg/ µL	2 µL	20 µL
SacI	0.5 µL	5 µL
SpeI	0.5 µL	5 µL

Tab 2.4.7.2, Reaction mix for plasmid and inserts enzyme digestion.

DNA digested	DNA concentration (ng/µL)	Volume of DNA needed to make 1µg DNA (µL)	Volume of sterile MQ water (µL)	Volume Master mix (µL)	Total (µL)
Plasmid S24	440	2.3	12.7	5	20
rhlA promoter	59	15	0	5	20
rhlB promoter	81	12.5	2.5	5	20
rhlI promoter	67	15	0	5	20
pqsA promoter	64	15	0	5	20
pqsB promoter	65	15	0	5	20

2.4.8. Visualisation of plasmid and inserts on agarose gel

A 1% agarose gel was prepared as described in 2.4.2.1. 10µL of insert and 0.5 µL plasmid sample plus 9.5 µL of water was mixed to 2µL and of 6X loading dye. The gel was run at 125V for 25 minutes. The inserts bands were extracted from the gel by using QIAquick Gel Extraction Kit, eluted in 30µL EB buffer and stored at -20°C. For the plasmid bands, due to the presence of unexpected bands, the gel was let to run up to 60 minutes, and the desired bands subsequently extracted using QIAquick Gel Extraction Kit, eluted in 60µL EB buffer, re-run onto an agarose gel, extracted and purified again using the same kit.

2.4.9 Dephosphorylation

Dephosphorylation was performed, to prevent relegation of linearized plasmid DNA. A reaction of 20µL was prepared by adding 17µL of digested plasmid DNA, 1µL FastAP Thermosensitive Alkaline Phosphatase from Thermo Fisher and 2µL of FastAP Thermosensitive Alkaline Phosphatase 10X Reaction Buffer, incubated for 1 hour at 37°C, then the heated up at 75°C to deactivate the enzyme for 5 minutes.

2.4.10 Ligation

Plasmid DNA was ligated to the inserts by using Promega T4 ligase, to allow phosphodiester bond formation between the complementary DNA strands. A reaction of 10µL was made by adding 2.3µL of plasmid DNA, 2.3µL of each insert, 5µL of Promega 2X ligation buffer and 0.4µL T4 ligase. A negative control was set up by adding 2.3µL sterile water instead of inserts. The mix was incubated at room temperature for 1 hour.

2.4.11 Transformation into *E. coli* DH10β competent cells

E. coli DH10β competent cells stored at –80 °C were defrosted on ice for 10 minutes. 5µL transformation mix was added to 50µL competent cells in a falcon tube, genlty mixed by flicking the bottom of the tube and incubated on ice for 20 minutes. Each tube was heat shocked by placing the tube in a 42°C water bath for 40 seconds and then incubated on ice for 2 minutes. 100 µL of LB was added to the cells and then left at 37 ˚C for 60 minutes, on shaker at 180 rmp. This step (outgrowth step) allows the bacteria to generate the antibiotic resistance protein encoded in the plasmid backbone. The mix was then spread onto LB agar plate with 50 µg/mL tetracycline and incubated overnight at 37°C. A negative control was set up by adding the ligation mix without insert to the plasmid, and one positive control with the plasmid only.

2.5 Biofilm assay

The crystal violet assay is based on staining bacterial cells that are adherent to the wells of a microtiter plate. *P. aeruginosa* and *E.coli* BL21 were grown in LB for 18 hours at 37°C with rotary shaking. The cultures were then centrifuged at 1300 rpm and washed with phosphate buffered saline (PBS) three times, to remove any antibiotic trace, then resuspended in Mueller Hinton broth (M-HB). Cultures were then diluted to a final OD600 of 0.2. An aliquot of 10 µl of each cultures was added to each well of a 96 well microtiter plate, mixed with 190 µl of M-HB, making three replicates for each condition. As control well, 200 µl of M-HB was used without adding the inoculum. After a static incubation at 37°C for two hours, the supernatant was gently removed

with a multichannel pipette, in order to get rid of non-adherent cells and the media was replaced with fresh M-HB. A second 24 hours incubation followed (37°C, without shaking). To measure the amount of biofilm formed, the supernatant was removed and the plate gently washed into a tub of water by submerging it and shaking out water two times. 125 µl of crystal violet solution (0.1% W/v in ethanol) was added to each well, incubated for 15 mins at room temperature and then removed by washing the plate in water, 3-4 times. Water was shaken out and the plate left to dry for few hours until dry, after which 125 µl acetic acid (30% v/v in H20) was added into the wells to solubilise the crystal violet stain. After 15 mins incubation at room temperature, 125 µl of the mixture from each well was transferred into a new fresh microtiter plate and Absorbance at 550 nm was taken by using a plate reader and acetic acid as a blank.

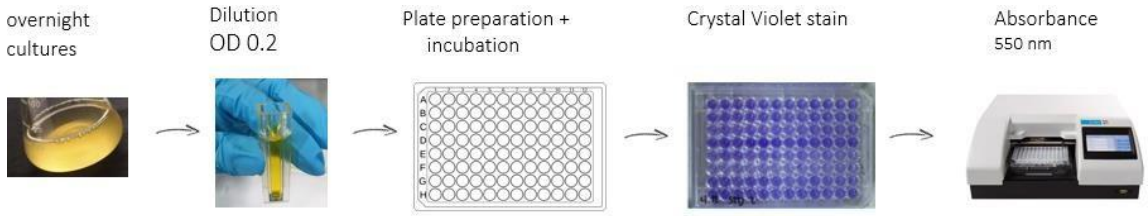


Fig. 2.5 Schematic of a biofilm assay workflow. *An overnight bacterial LB suspension was diluted to OD 0.2 and aliquoted into a microtiter plate and bacteria left to grow for two hours; supernatant cells were removed by numerous washes and remaining biofilm stained with crystal violet for subsequent Absorbance readout. Created on words.*

2.5.1 Biofilm assay with biofilm inhibitors

Test compound 4-Nitropyridine-N-oxide NPO was dissolved in DMSO to achieve concentrations ranging 0.01-1M and consequently diluted down with bacterial cultures to achieve concentrations ranging from 0.1–10 mM while keeping DMSO at a maximum of 1% (v/v). An aliquot of 100 µl of this cell/compound mixture was then added to three separate wells in a 96-well microplate for replicate testing. For control wells (no inhibitor), DMSO was added to the inocula instead of test compounds, to a final concentration of 1% (v/v). The same procedure described above was followed in order to get GFP fluorescence measurements and optical density measurements at OD600.

2.6 Infection assay with biofilm inhibitors

The same protocol as described for liquid survival assay in chapter 2.2.2.2 was performed with the addition of test compound 4-Nitropyridine-N-oxide NPO. NPO was initially dissolved in DMSO to achieve concentrations ranging 0.01-1M, and then mixed to overnight-grown bacterial cultures of *E. coli* OP50 and *P. aeruginosa* PA14 with concentrations ranging 0.1– 10 mM while keeping DMSO at a maximum of 1% (v/v). 79 µl of M9 were added to 20 µl of bacterial culture and 1 µl of test compound, to make up for a total of 100 µl of media in each well. Control wells were set up without inhibitor. *glp-1 C. elegans* were initially grown onto agar plate at 20°C, fed with *E. coli* OP50; L4 worms were picked with a sterile platinum wire pick and transferred into each well. After 3 days of incubation at 25°C, dead worms were counted for each condition. Worms which did not display any movement were counted as dead.

2.7 Fluorescence assay

P. aeruginosa and *E. coli* BL21 were grown in LB for 18 hours at 37°C with rotary shaking. The cultures were then centrifuged at 1300 rpm and washed with phosphate buffered saline (PBS) three times, to remove any antibiotic trace, then resuspended in Mueller Hinton broth (M-HB). Cultures were then diluted to a final OD600 of 0.2. An aliquot of 10 µl of each cultures was added to each well of a 96 well microtiter plate, mixed with 190 µl of M-HB, making three replicates for each condition. As control well, 200 µl of M-HB was used without adding the inoculum. After a static incubation at 37°C for two hours, the supernatant was gently removed with a multichannel pipette, to get rid of non-adherent cells and the media was replaced with fresh M-HB. A second 24 hours incubation followed (37°C, without shaking) before taking GFP fluorescence measurements by using a microplate spectrofluorometer reader.

2.8 DMR epifluorescence microscopy

To visualize fluorescence expressed within *C. elegans* gut, a thin layer of 2.5% agarose was made by squashing a warmed-up droplet in between two clean slides. Once solidified, one slide was removed and 5µl drop of Tetramisole, diluted 1:4 with M9, was added to the top of the agarose. 10 worms from each condition of interest were transferred from the agar plate onto the tetramisole drop, which immobilised them. With the aid of an eyelash pick, the worms were gently put close together and a cover lid was then positioned on top, ready for imaging. Imaging was carried out at 10X and 40X, using a green filter.

2.9 statistical analysis

Statistical analysis was carried out using excel and GraphPad Prism. One-way ANOVA test was carried out to compare means of two or more study groups to determine statistical significance, with comparison to a control group. Particularly, this test was used for the fluorescence detection and biofilm quantification results from the microtiter plate reader assays and refer to comparison with E.coli BL21, using 0.05 as threshold for rejection of the null hypothesis. Therefore, a p-value<0.05 is considered as moderately significant (*), a p-value<0.01 is considered strongly significant (**), and a p-value<0.001 is considered robustly significant(***). To compare survival distributions of different groups, Statistical significance was also calculated using unpaired t-test and Log-rank test, assuming 0.05 as threshold for rejection of the null hypothesis. Therefore, a p-value<0.05 is considered as moderately significant (*), a p-value<0.01 is considered strongly significant (**), and a p-value<0.001 is considered robustly significant(***). Standard deviation was used to measure the dispersion of the datasets; this was calculated using GraphPad Prism and represents the square root of the variance from the mean between biological repeats, indicated by error bars.

CHAPTER 3
RESULTS: building an in-vivo biofilm-specific reagent

3.1 Cloning of biofilm-associated *rhlA, rhlB, rhlI, PqsA* and *pqsB* genes' promoters into a vector

3.1.1 Isolation of Plasmid mini CTX from *E.coli*

With the goal of making an in-vivo biofilm reporter, a cloning strategy was executed to make a reagent expressing fluorophores under expression of genes involved in biofilm formation. The promoters of biofilm-related genes (*rhlA, rhlB, rhlI, PqsA* and *pqSB)* were designated to be cloned into a vector, which could be transformed into *Pseudomonas aeruginosa* cells. The overall goal would then be to infect *C. elegans* with the transgenic bacteria expressing biofilm-driven fluorescent markers.

In order to clone the promoter region of the genes *rhlA, rhlB, rhlI, pqsA* and *pqsB* into the miniCTX1 vector, cultures of miniCTX1 in *E. coli* were miniprepped to isolate the plasmid and the quality of the vector was assessed. Absorbance at 260/280 of the sample of 1.69 indicated DNA purity, while the plasmid concentration was of 150 ng/µL. Results from the agarose gel confirmed that plasmid isolation was successful, but it also flagged a partial digestion of the vector, as shown in fig.3.1.1. As shown in fig. 3.1.1 A and B, upon restriction digestion with SpeI + SacI (lane 3), BamHI + SacI (lane 4), BamHI + SpeI (lane 5) and BamHI only (lane 6), the expected band sizes were of 25bp + 7800 bp, 711bp + 792bp + 6300 bp, 711bp + 792bp + 6300 bp and 792 bp + 7000 bp respectively. As shown in fig 3.1.1 C, the obtained bands had different sizes as what expected: lane 3 had three bands of 1.2, 3 and 6000 bp, while only one band (7852 bp) for the linearized plasmid should have been visible (the 25 bp band would be too small to be visible); finally, lane 4, 5 and 6 had 3 bands of incorrect sizes too (1.2, 3 and 6000 bp). As expected, lane 2 showed two bands: undigested plasmids normally yields two or more bands in their gel lane, due to their non-linearity; these bands represent the secondary structure of the plasmid, which often appear in two forms, a supercoiled circular DNA which migrates faster and further down in the gel and an open circular form, which moves slower and therefore appear higher in the gel. Unfortunately, the same bands

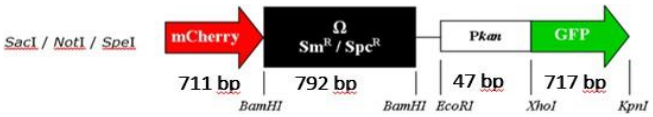
persisted in most of the plasmid digests (from lane 3-6), showing a lot of residual undigested plasmid and poor digestion.

To conclude, the gel highlighted a problem with the vector, which did not seem to be digested as expected. Many could be the causes, such as plasmid recombination, sequence mutations, plasmid toxicity for the transformant cells (further discussed in chapter 5.5).

A

gel lane	Restriction enzymes	Band size
1	ladder	
2	Undigested	2 or 3 bands expected
3	SpeI +SacI	25bp + 7852 bp
4	BamHI + SacI	711bp + 792bp + 6300 bp
5	BamHI + SpeI	711bp + 792bp + 6300 bp
6	BamHI only	792 bp +7000 bp

B



C

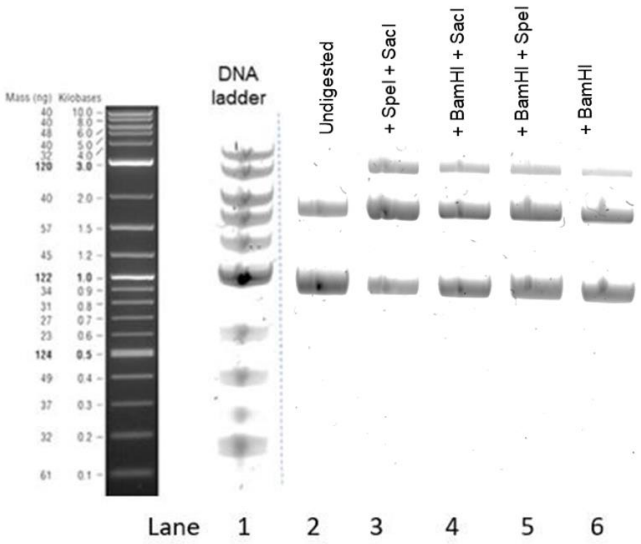


Fig 3.1.1. Quality check of the plasmid. A) *Table showing the expected band size for each restriction enzymes used.* ***B) Diagram of the plasmid construct****. Diagram showing each segment length of the plasmid construct. mCherry and GFP are shown in red and green respectively, along with restriction sites. Obtained from the Hall lab (University of kent), made on Word.* ***C) Dna agarose gel showing the miniprep yields of miniCTX1 and plasmid size check by restriction digestion.*** *A) Lane 1, DNA ladder, lane 2 undigested plasmid, lane 3 SpecI+ SacI plasmid digestion, lane 3 BamHI + SpeI plasmid restriction digestion, lane 4 BamHI+ SacI restriction digestion, lane 5 BamHI + SpeI restriction digestion and lane 6 BamHI only.*

3.1.2 Inserts amplification from *PA14 gDNA*

As described in chapter 2.4.5, after PCR amplification of the promoter regions of *rhlA, rhlB, rhlI, pqsA* and *pqsB* genes using genomic DNA previously isolated from *Pseudomonas aeruginosa PA14,* the inserts were gel purified, to confirm amplification and correct size of 536bp by electrophoresis (chapter 2.4.6). As shown in fig. 3.1.2, bands were 536bps in size, as expected.

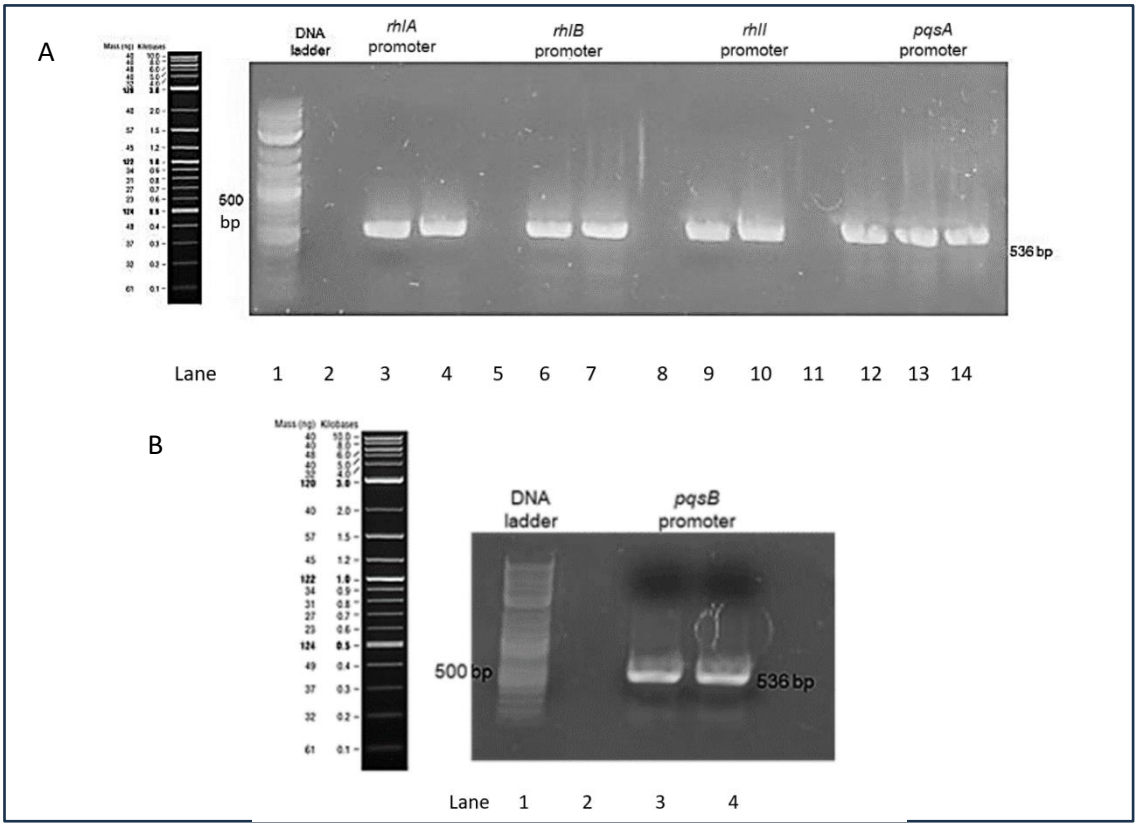


Fig 3.1.2. 1% gel electrophoresis of rhlA, rhlB, rhlI, pqsA and pqsB promoters*. A) Lane 1 DNA ladder, lane 2 empty, lane 3 and 4 rhlA promoter (two repeats), lane 5 empty, lane 6 and 7 rhlB promoter (two repeats), lane 8 empty, lane 9 and 10 rhlI promoter (two repeats), lane 11 empty, lane 12-14 pqsA promoter (three repeats). Bands were the expected size of 536bps. B) Lane 1 DNA ladder, lane 2 empty, lane 3 and 4 pqsB promoter (two repeats). Bands were the expected size of 536bps.*

3.1.3 Restriction digest of plasmid and inserts

The plasmid vector and the inserts were digested with restriction enzymes SacI and SpeI to create sticky ends and to make the vector compatible with the inserts. As shown in *fig. 3.1.3* the promoter bands were the correct size, but the digested plasmid contained three bands instead of a single band, similarly to what was obtained for the quality check of the plasmid in chapter 3.1.1. This was clearly caused by the presence of residual undigested plasmid, due to partial digestion.

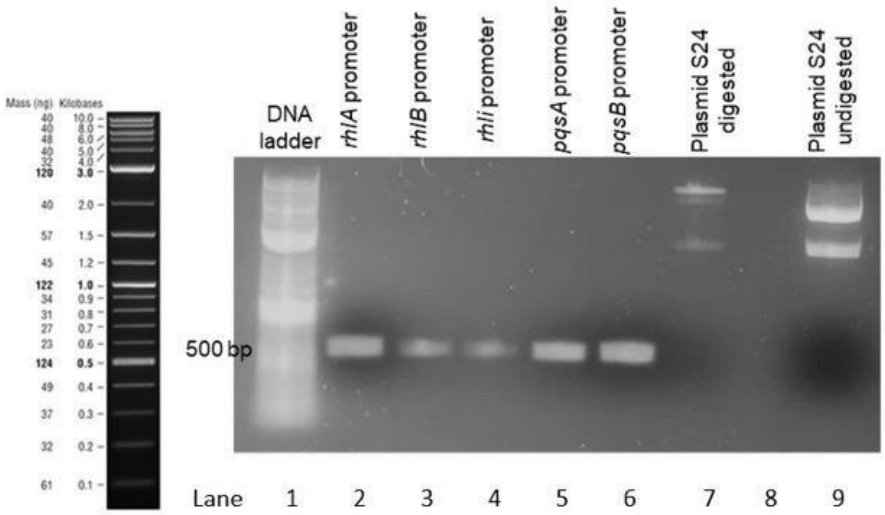


Fig. 3.1.3 Visualisation of Plasmid and Inserts after restriction digest on 1% agarose gel. *Lane 1 DNA ladder, lane 2 rhlA promoter digest, lane 3 rhlB promoter digest, lane 4 rhlI promoter digest, lane 5 pqsA promoter digest, lane 6 pqsB promoter digest, lane 7 plasmid digest, lane 8 empty, lane 9 undigested plasmid. Lane 7 shows partial digestion of the plasmid, indicated by the presence of two unexpected bands, which also appear in lane 9.*

3.1.4 Cloning troubleshooting

In order to proceed with the cloning, a new digestion of the plasmid with SacI and SpeI was performed and re-run on agarose gel for up to one hour at 125V, in order to better separate the bands and subsequently extract the band of the correct size of 7800 bs (indicated by an arrow in fig. 3.1.4 A). Various repeats were loaded into the gel, as shown underneath. Four of the extracted bands were put onto 1 column, achieving a DNA concentration of 22 ng/µL, while 2 of the bands were put onto a second gel extraction column, obtaining a DNA concentration of 3 ng/µL. As shown in fig. 3.1.4 B, a new gel was run to check that only one band was visible. The obtained band was extracted and stored at -20°C to proceed to the next step.

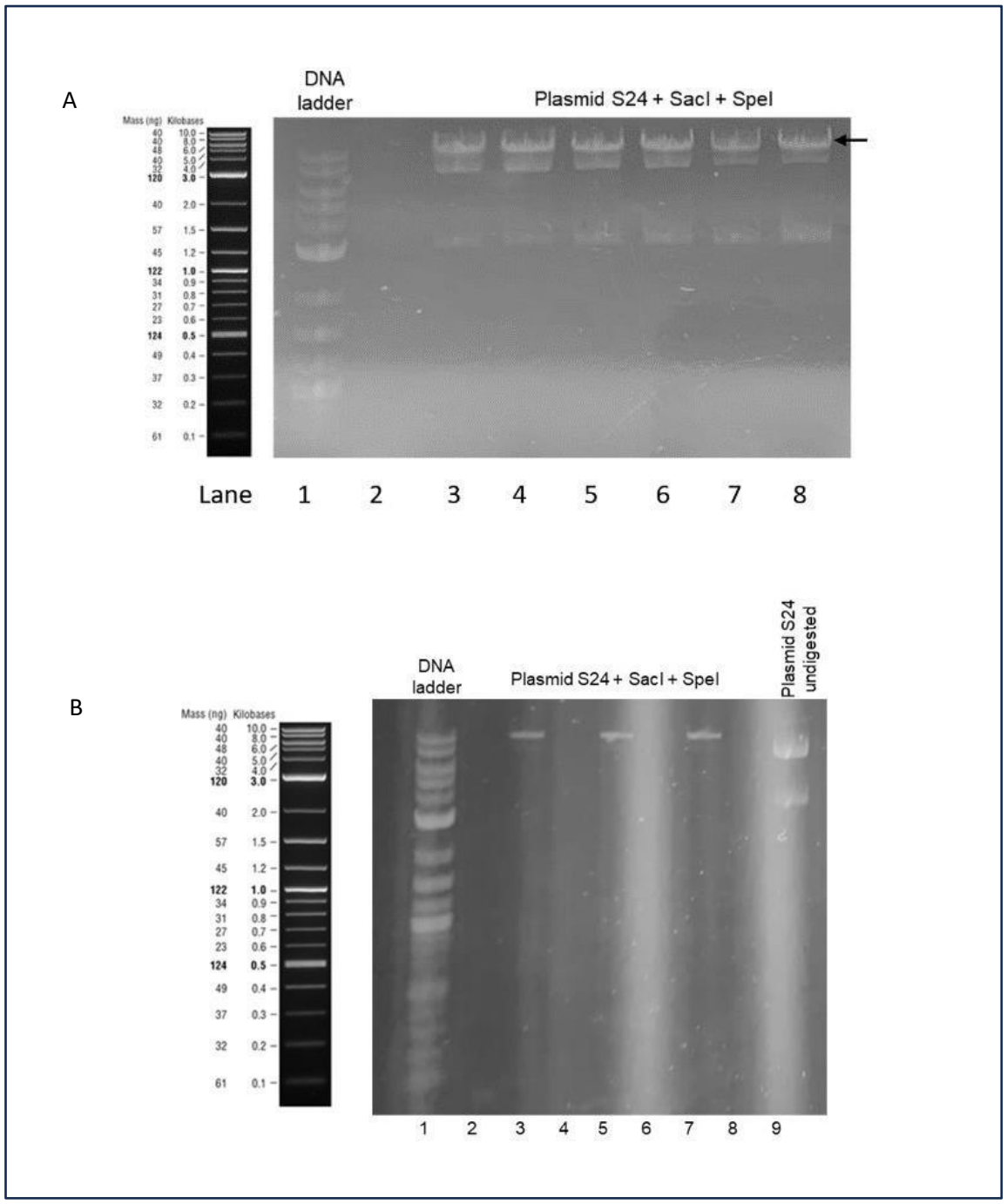
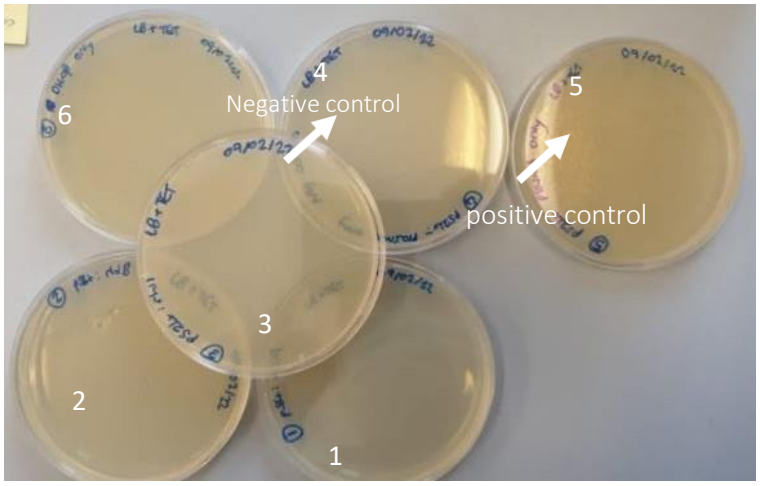


Fig. 3.1.4 Plasmid S24 digestions with SacI and SpeI. *A) Lane 1 DNA ladder, lane 2 empty, lane 3-8 plasmid digest. The arrow indicates the band of interest which was extracted from the gel. B) second gel electrophoresis of the plasmid bands, extracted in A. Lane 1, DNA ladder, lane 2 empty, lane 3, plasmid digest (3 ng/µL), lane 5,7 plasmid digest (22 ng/µL), lane 9 undigested plasmid.*

3.1.5 Unsuccessful transformation into *E. coli* DH10β competent cells and final conclusions

After dephosphorylation and ligation of the plasmid to the inserts (chapter 2.4.9/10), transformation into *E. coli* DH10β competent cells was performed. Unfortunately, the transformation was unsuccessful: as shown in fig.3.1.5, the plates with the ligation mix did not have any colony (fig. 3.1.5 A) and only the positive control plate (plasmid only, added to *E. Coli* DH10β competent cells) had bacterial colonies (fig. 3.1.5 B). The fact that the positive control plate had colonies reassured of the accuracy of the protocol carried out but, unfortunately, the lack of colonies from the ligation mix also confirmed that the cloning did not happen, probably due to the failed initial digest, flagged by the previous agarose gels.

A



B

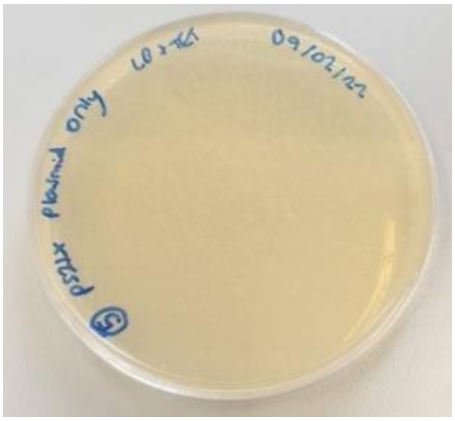


Fig. 3.1.5. Transformation with E. coli DH10β competent cells. *A) Plate 1: ligation mix of pS24+rhlA insert; Plate2: ligation mix of pS24+rhlB insert; Plate 3: ligation mix of pS24+rhlI insert; Plate 4: negative control, digested plasmid only, no inserts; Plate 5: positive control, undigested plasmid only; Plate 6: E. coli DH10β competent cells only. B) Only the positive control had colonies.*

3.1.6 Unsuccessful cloning and final conclusions

Overall, the cloning revealed to be very challenging. The electrophoresis gels shown in chapters 3.1.1-3.1.4 flagged a problem with the vector digestion, which only appeared to happen partially. The pattern of bands in the undigested plasmid lane (fig. 3.1.1 C) are representative of the secondary structure of the circular plasmid, which often shows up in two forms: a compact supercoiled covalently closed DNA structure, which moves faster and normally migrates further down in the gel; a second open circular form, which moves slower through the pores of the gel matrix and therefore appear higher in the gel. On the contrary, a completely digested plasmid, shows up a single band in its gel lane, due to linearity of the plasmid. Unfortunately, the digested plasmid lanes showed three bands, two of which of the same size as those in the undigested plasmid lane, showing that the digestion only partially worked. This might have compromised the entire cloning experiment, resulting in the absence of any bacterial colony, after transformation into *E. coli* DH10β competent cells.

Due to lack of time, the process could not be revised any further and the project moved on for another direction, as explained in the next chapter, where seven transgenic fluorescent Pseudomonas strains were sourced from different research groups and evaluated for their suitability in this biofilm study.

CHAPTER 4

RESULTS: evaluating *P. aeruginosa* reporter strains for biofilm studies

4.1 Sourcing of *P. aeruginosa* reporter strains

Due to the difficulties encountered in cloning the reagent of interest, and due to lack of time to further troubleshoot, an alternative strategy was adopted, to conduct the project.

Seven transgenic, fluorescent Pseudomonas strains were sourced from different research groups, to evaluate their application to in vitro biofilm studies and in vivo biofilm monitoring, in *C. elegans.* The obtained reporter strains express fluorophores under the expression of promoters of genes related to quorum sensing communication, a cell-to-cell communication process which relies on signaling molecules (autoinducers). A biofilm assay was initially employed to evaluate in vitro biofilm production of each strain, and subsequently visualised into *C. elegans* for in vivo analysis.

4.1.1 Targeting the LasI/R and RhlI/R pathway

The lasI/R pathway relies on the autoinducer 3OC12-homoserine lactone, which expression is under the *LasI* gene control. Biofilms of *lasI* mutants appear less uniform (17) confirming the role of the system in biofilm formation and maturation. The RhlI/R pathway relies on the autoinducer C4-homoserine lactone. This system has a role in virulence and biofilm development, and the QS receptor RhlR plays a major role in it (18). The reagents obtained from Gary Robinson at University of Kent, are three mutants of the strain PAO1, which lacked either LasI gene or RhlI, or both, and hosted a plasmid with GFP reporter under the lac promoter. (43) On the other hand, reagents obtained from Bonnie Bassler lab at Princeton University, used a plasmid in PA14 background with mNeon green fluorescent transmission reporter fusion to the rhlA promoter, expression of which is regulated by the gene *rhlR*. (44) The same assays described above were carried out on these reagents. These reagents were acquired due to their mutations in the *las* and *rhl* quorum sensing systems, to evaluate their biofilm production and suitability for biofilm visualization in vitro and in vivo*;* these were used to carry out both in vitro biofilm assay using a crystal violet staining and in vivo studies on *C. elegans* to evaluate the intestinal colonization of the warms and detect any

presence of biofilm using DMR microscopy. Each transgenic strain was compared to both E.coliBL21 which does not form biofilm and its respective wildtype PA strain (PA01 and PA14). The strains used and purpose of the experiment are listed in tab 4.1.1.

Tab 4.1.1. Transgenic, fluorescent strains targeting LasI/R and RhlI/R

Strain name	PA background	Species	Origin	Test	Purpose	Target QS pathway
PA01 wild type		*Pseudomonas sp.*		Biofilm/fluorescence assay/ C.elegans survival assay	Control strain for PA transgenic strains	
PDO-100 PAO1 Δ*RhlI*; with GFP expressed constitutively from lac promoter (plasmid pTdK-GFP)	*PA01*	*Pseudomonas sp.*	Gary Robinson, University of Kent	Biofilm/fluorescence assay/ C.elegans survival assay	Test importance of RhlI in biofilm and test suitability for biofilm detection assay	*Rhl* system
PAO-JP1 PAO1 Δ*lasI* with GFP expressed constitutively from *lac* promoter (plasmid pTdK-GFP)	PA01	*Pseudomonas sp.*		Biofilm/fluorescence assay/ C.elegans survival assay	Test importance of lasI in biofilm and test suitability for biofilm detection assay	*Las* system
PAO-JP2; PAO1 Δ*rhlI* Δ*lasI* with pTdK-GFP	PA01	*Pseudomonas sp.*		Biofilm/fluorescence assay/ C.elegans survival assay	Test importance of lasI and RhlI in biofilm and test suitability for biofilm detection assay	*Las* and *Rhl* systems
Sm381 - *PrhlA-mNeonGreen*	*PA14*	*Pseudomonas sp.*	Bonnie Bassler, Princeton University	Biofilm/fluorescence assay/ C.elegans survival assay	Test importance of RhlA in biofilm and test suitability for biofilm detection assay	*Rhl* system
Sm383 - Δ*rhlR PrhlA-mNeonGreen*	PA14	*Pseudomonas sp.*		Biofilm/fluorescence assay/ C.elegans survival assay	Control strain (does not form biofilm)	*Rhl* system
E. coli BL21		*Escherichia coli*	Becky Hall, University of Kent	Biofilm/fluorescence assay/ C.elegans survival assay	Control strain (does not form biofilm)	

4.1.2 Targeting the Type III secretion system

Reporter strains of the Exoenzyme S, a virulence factor and structural protein of the Type III secretion system, were also used in the study. The system is used by gram-negative bacteria to injects toxins through a needle-like apparatus, and there seems to be a link with biofilm in certain conditions, particularly for epithelial biofilm formation.(45) The strains obtained by Becky Hall laboratory at University of Kent, contain a plasmid pJNE05 which encodes for the effector protein ExoS fused to GFP reporter, into PA14 background and the aflagellate laboratory strain PA103. These reagents were acquired due to their expression of ExoS protein, linked to the secretory pathway III, which has been shown to contribute towards biofilm formation; therefore, they were picked to evaluate their biofilm production and suitability of the ExoS fused GFP reporter for biofilm visualization in vitro and in vivo. The reagents were used to carry out both in vitro biofilm assay using a crystal violet staining and in vivo studies on *C. elegans* to evaluate the intestinal colonization of the warms and detect any presence of biofilm using DMR microscopy. Each transgenic strain was compared to both E.coliBL21 which does not form biofilm and its respective wildtype PA strain (PA14 and PA103). The strains used are listed in tab 4.1.2.

Tab 4.1.2. Transgenic, fluorescent strains targeting Type III secretory pathway.

Strain name	PA background	species	Origin	test	purpose	target
PA14 wild type				Biofilm/fluorescence assay/ C.elegans survival assay	Control for PA14 mutant	
PA14 with *ExoS* GFP reporter (plasmid pJNE05)	*PA14*	*Pseudomonas sp.*	Becky Hall, University of Kent	Biofilm/fluorescence assay/ C.elegans survival assay		Type III secretion system
PA103 wild type	PA103			Biofilm/fluorescence assay/ C.elegans survival assay	Control for PA103 mutant	
PA103 with *ExoS* GFP reporter (plasmid pJNE05)				Biofilm/fluorescence assay/ C.elegans survival assay		Type III secretion system
E. coli BL21		*Escherichia coli*	Becky Hall, University of Kent	Biofilm/fluorescence assay/ C.elegans survival assay	Control strain	

4.2. In vitro biofilm studies

4.2.1. LasI/R and RhlI/R reporter strains produce biofilm in vitro and could be used for biofilm studies

To measure the ability of the above strains to form biofilm, staining with Crystal Violet (CV) was performed in a microtiter plate, along with a biofilm-incompetent control strain (*E. coli* BL21). CV is employed to stain peptidoglycan in bacterial cell walls, therefore enabling biofilm quantification by absorbance measurements in a plate reader.

We found that, in comparison to the control EcoliBL21, PA01wild type has similar ability to form biofilm, as shown in fig. *4.2.1 A*; similarly, the mutant with double gene knockout *ΔlasI;ΔrhlI* had low levels of biofilm formation, suggesting that these genes may have an important role in biofilm formation but only when acting in synergy. On the contrary, *RhlI* or *LasI* single gene knockout did not seem to affect biofilm formation at all: the *ΔrhlI* strain yielded a significant two-fold increased CV stain compared to both the control *E. coli BL21* and wildtype *PA01*; *ΔlasI* also had higher CV staining in comparison to *PA01,* although not significant.

Very interestingly, we also found that the *ΔrhlR* mutant had decreased biofilm forming ability, as shown in fig. *4.2.1 B,* with one-fold reduced absorbance, when compared to the non-mutant reporter strain *prhlA-mNeonGreen*.

Overall, when looking at *P. aeruginosa* pTdK-GFP reporter strains, these results suggest that RhlI and LasI are not the major player in *Pseudomonas* biofilm formation, but they may have an impact on biofilm formation when synergistically switched on. Moreover, the gene *rhlR* seems to play a role in biofilm formation of *P. aeruginosa*.

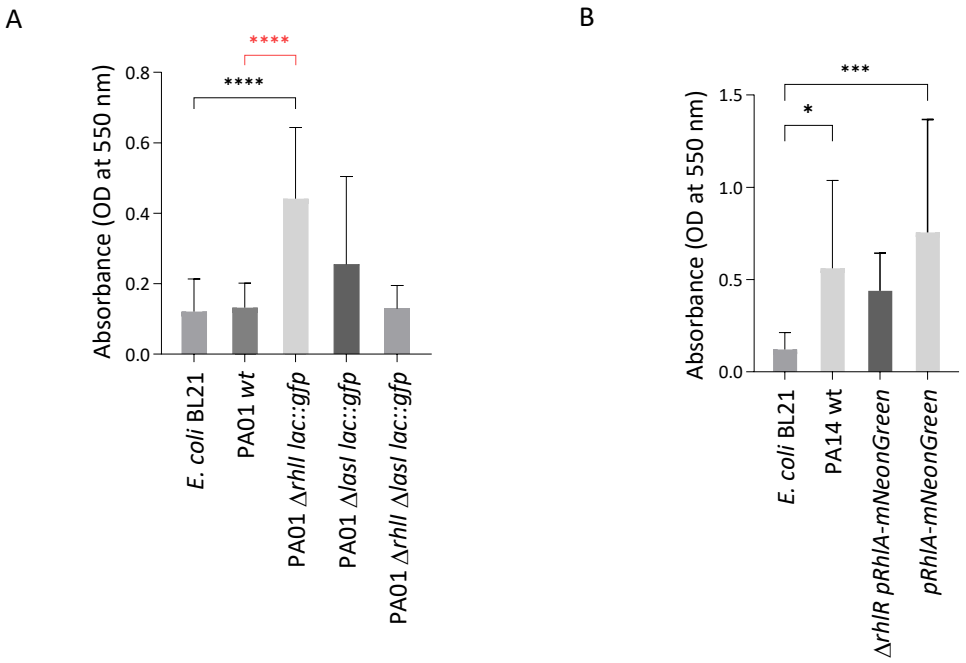


***Fig. 4.2.1 Biofilm detection of P. aeruginosa pTdK-GFP reporter* strains.**
Crystal Violet staining measured at OD 550 nm in microtiter plate for quantification of biofilm. For each bacterial strain three biological repeats were performed. Error bars represent standard deviation of the replicates and statistical significance refers to comparison to the control E. coli BL21 in black color, and each mutant strains was also compared to the respective wild type, shown in orange color. (One-way ANOVA, *p <0.05, **p <0.01, ***p <0.0001) *Panel A) showing strains: PAO1 wild type, PDO-100 PAO1 ΔRhlI; with GFP expressed constitutively from lac promoter (plasmid pTdK-GFP), PAO-JP1 PAO1 ΔlasI with GFP expressed constitutively from lac promoter (plasmid pTdK- GFP), PAO-JP2; PAO1 ΔrhlI ΔlasI with pTdK-GFP. Panel B) showing E.Coli BL21, PA14wt, Sm383 - ΔrhlR PrhlA-mNeonGreen and Sm381 - prhlA-mNeonGreen.*

4.2.2. LasI/R and RhlI/R reporter strains exhibit fluorescence expression in vitro: using fluorescence to determine biofilm presence

We used a fluorescence based assay to test whether fluorescence expression could be used to detect and quantify biofilm in the reporter strains. Fluorescence was measured in a microtiter plate reader, after growing the cultures on a solid surface and washing planktonic cells away. As shown in fig.4.2.2, *E. coli* BL21 did not show any fluorescence and PA01 also showed low levels of fluorescence, even though slightly higher than the control, probably due to autofluorescence (Fig.4.2.2 A). Confirming the biofilm assay, *ΔrhlI lac::gfp* had 6-fold higher fluorescence intensity than the control *E. coli and control* PA01wt (Fig.4.2.2 B), much higher when compared to *ΔlasI lac::gfp* and *ΔlasI ΔrhlI lac::gfp*. Contradicting the biofilm assay, the *ΔlasI ΔrhlI lac::gfp* also exhibited significantly high levels of fluorescence, compared to E.coliBL21 and PA01 wt, even though lower levels of fluorescence were yielded, in comparison with the single mutants, reinforcing the hypothesis of a synergistic effect of the two genes in biofilm formation. On the other hand, the *pRhlA-NeonGreen* reporter strains exhibited fluorescence which was significantly reduced 3.5 fold in the *ΔrhlA* mutant, reinforcing the biofilm assay results.

Overall, these results show that a fluorescence detection method can be used in vitro for biofilm detection, due to similarity of the results with the biofilm assay described in the previous chapter; however, *P. aeruginosa* autofluorescence patterns could be observed and need to be taken into account, when carrying out this type of assay.

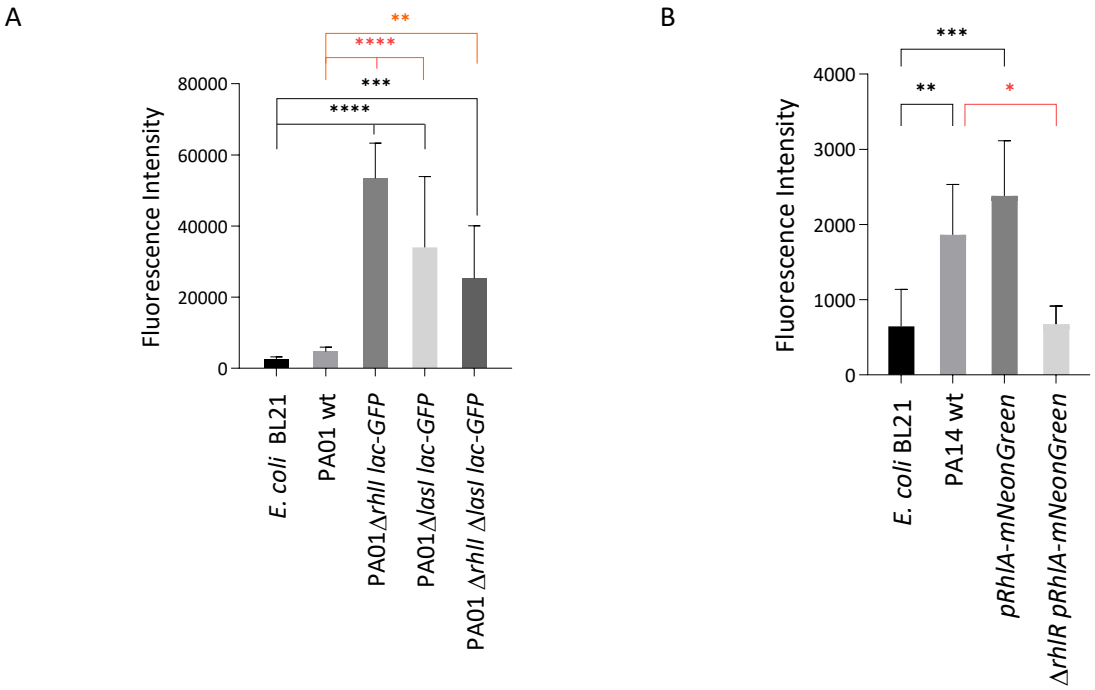


Fig. 4.2.2 Fluorescence expression of P. aeruginosa biofilms in vitro. *GFP fluorescence was measured in microtiter plates to quantify biofilm formation. Three biological repeats were performed for each strain. Error bars represent standard deviation, statistical significance refers to comparison to the control E. coli BL21 in black color, and each mutant strains was also compared to the respective wild type, shown in orange color. . (*One-way ANOVA, *p <0.05, **p<0.01, ***p <0.0001) *Panel A) showing strains: E.coli BL21, PAO1 wild type, PDO-100 PAO1 ΔRhlI; with GFP expressed constitutively from lac promoter (plasmid pTdK-GFP), PAO-JP1 PAO1 ΔlasI with GFP expressed constitutively from lac promoter (plasmid pTdK-GFP), PAO-JP2; PAO1 ΔrhlI ΔlasI with pTdK-GFP. Panel B) showing E.coli BL21, PA14 wild type, Sm381 - PrhlA-mNeonGreen, Sm383 - ΔrhlR PrhlA-mNeonGreen*

4.2.3. Exoenzyme S reporter strains produce biofilm in vitro and could be used for biofilm studies

Results obtained from the biofilm assay showed that, compared to a biofilm-incompetent control strain (*E. coli* BL21), PA14 WT has increased ability to form biofilm, represented by stronger CV staining and higher absorbance measurement. (fig. 4.2.3); the same was noticed in the transgenic reporter strain PA14 with pJNE05, the absorbance of which had a significantly higher measurement. On the contrary, PA103 has reduced ability to form biofilm, such as its transgenic counterpart, PA103 with pJNE05.

These results show that PA14 background is more suitable for biofilm assays, due to the ability of this strain to form biofilm, as reflected by the higher absorbance levels, when compared to PA01. High levels of absorbance for PA14 with pJNE05 also show the importance of the Type III secretion system for biofilm formation.

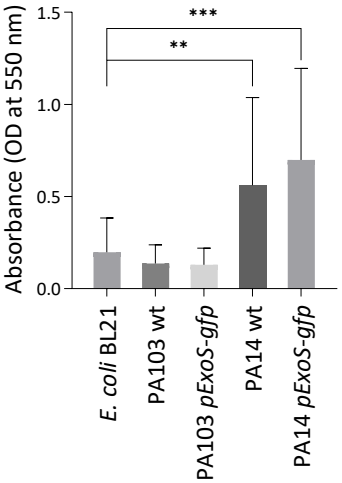
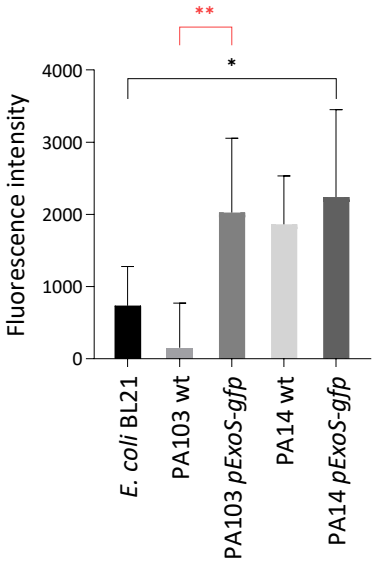


Fig. 4.2.3. biofilm detection in P. aeruginosa pExoS reporter strains. *Crystal Violet staining measured at OD 550 nm in microtiter plate for quantification of biofilm. For each bacterial strain three biological repeats were performed. Error bars represent standard deviation of the replicates and statistical significance refers to comparison to the control E. coli BL21. (*One-way ANOVA, *p <0.05, **p <0.01,
***p <0.0001) *Panel showing strains: PA14 wild type, PA14 with ExoS GFP reporter (plasmid pJNE05), PA103 wild type, PA103 with ExoS GFP reporter (plasmid pJNE05), E. coli BL21.*

4.2.4 Exoenzyme S reporter strains fluorescence expression in vitro

Fluorescence measurements revealed that the biofilm-competent PA14 had some level of autofluorescence, similarly to what observed in PA01, as shown in fig.4.2.4. Nevertheless, both pExoS reporter strains (PA103 pExoS-GFP and PA14 pExoS-GFP) had significantly higher levels of fluorescence expression, compared to the control E.coli BL21, but only PA103 PA103 pExoS-GFP had significantly higher expression levels, when compared to its wildtype control strain. This contradicted the biofilm assay, where PA103 pExoS-GFP appeared to form low levels of biofilm, therefore more studies should be carried out on fluorescence assays for biofilm detection and correlation between fluorescence expression and biofilm production.



4.2.4 Fluorescence expression of P. aeruginosa pExoS reporter strains in vitro. *GFP fluorescence was measured in microtiter plates to quantify biofilm formation. Three biological repeats were performed for each strain. Error bars represent standard deviation, statistical significance refers to comparison with E. coli BL21. (*One-way ANOVA, *p <0.05, **p <0.01, ***p <0.0001) *Panel showing strains: PA14 wild type, PA14 with ExoS GFP reporter (plasmid pJNE05), PA103 wild type, PA103 with ExoS GFP reporter (plasmid pJNE05), E. coli BL21. A blank control is also shown in black.*

4.3 In vivo biofilm studies: *C. elegans* infection assay with *P. aeruginosa*

4.3.1 *C. elegans* infection from egg lay and L4

P. aeruginosa is a well-known biofilm former, reason why the bacterium was chosen to infect *C. elegans* and study biofilm formation on the nematode. As described in chapter 2.2.2, infection was performed by following two different protocols: an agar-based solid culture system and LB broth-based liquid culture system. Adults animals were initially cultivated on the non-pathogenic *E. coli* OP50 and subsequently, their progeny was transferred to *Pseudomonas* strains, either from egg stage (by bleaching adults) or at the end of development at larval stage L4, to evaluate the best protocol. The two laboratory Pseudomonas strains PA01 and PA14 were compared, to understand which strain was most suitable to establish an infection in *C. elegans*.

As expected, worms grown onto OP50 (control) remained alive throughout the 8 days of the experiment, in both conditions (egg-lay and L4), as shown in fig. 4.3.1. On the other hand, worms fed with both PA strains showed lower percentages of survival. Particularly, 50% of the worms fed with PA14 since egg stage (Fig. 4.3.1, A) were already dead by day 3 of the infection and 10% of those fed with PA01 died too. Survival rates decreased significantly by day 6, when only 10% and 80% of the worms had survived, when fed with PA14 and PA01 respectively. By day 8, all the worms fed with PA14 were dead, while 80% of those fed with PA01, remained alive.

On the contrary, animals fed with PA strains from L4 stage (Fig. 4.3.1, B) had better survival profiles, with more than 50% of the animals fed with PA14 still alive by day 6, and 20% still alive by day 8. When grown onto PA01, *C. elegans* showed similar % survival rates to the control group, with >90% of the worms still alive by day 8.

Furthermore, not only worms grown onto PA strains from eggs died quicker, but some of the eggs did not hatch at all, when left onto PA14 bacterial lawns; moreover, animals didn't seem to develop as well as those on the control, being smaller, maybe due to the animals avoiding the food.

For the purpose of this project, it was concluded that the worms would be infected at L4 stage, to ensure longer survival and better observations of the animals. Finally, when compared to the control *E. coli*, PA01 was well tolerated by the worms, as opposed to PA14, which killed the entire population within 8 days, for both egg-lay and L4 infections. For this reason, for future *C. elegans* infection models, it was decided to proceed with PA14 infection, which elicited a stronger response in the animal. For the same reason, PA14 was also chosen to extract genomic DNA for the isolation of genes promoters used in the cloning.

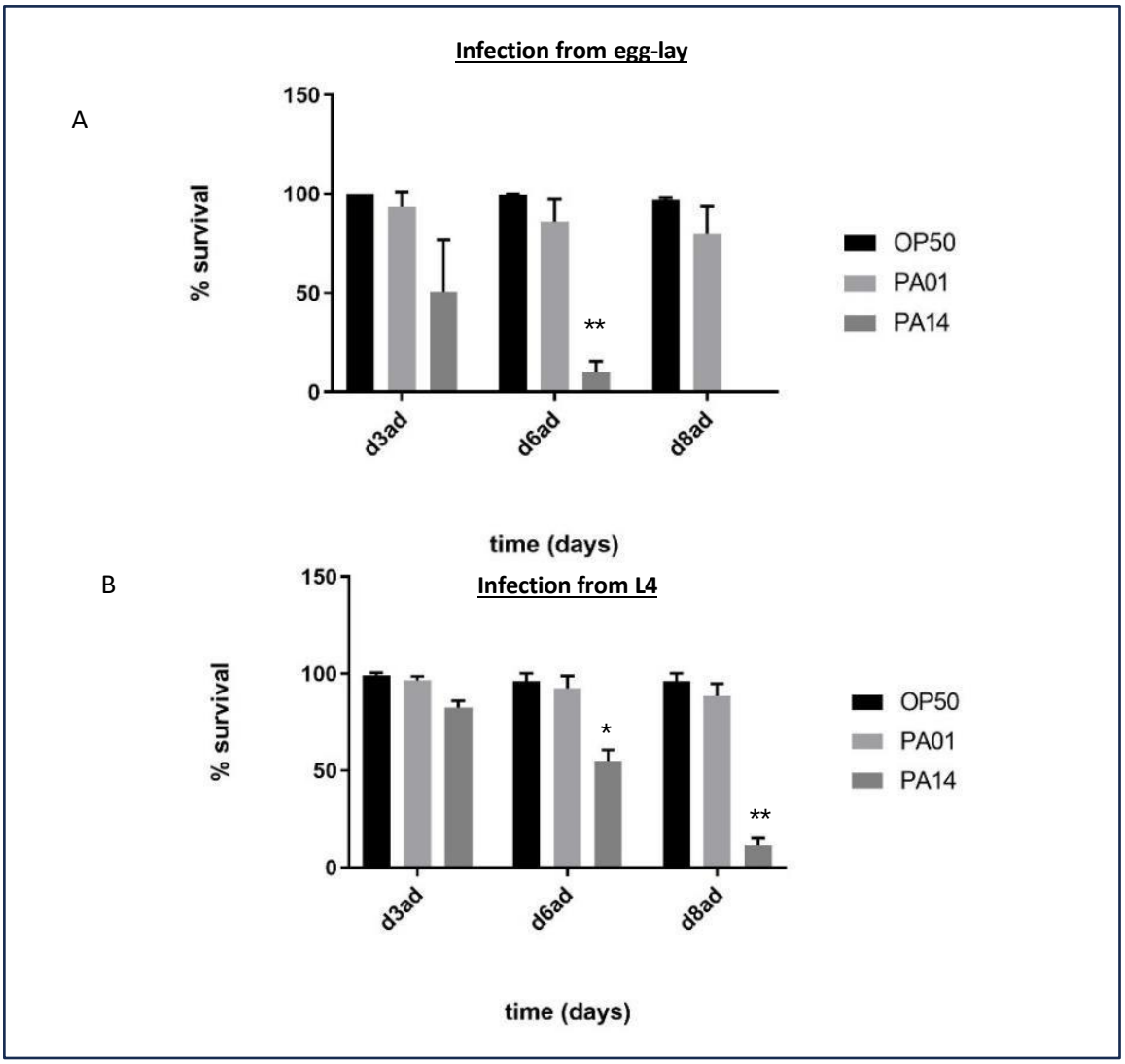


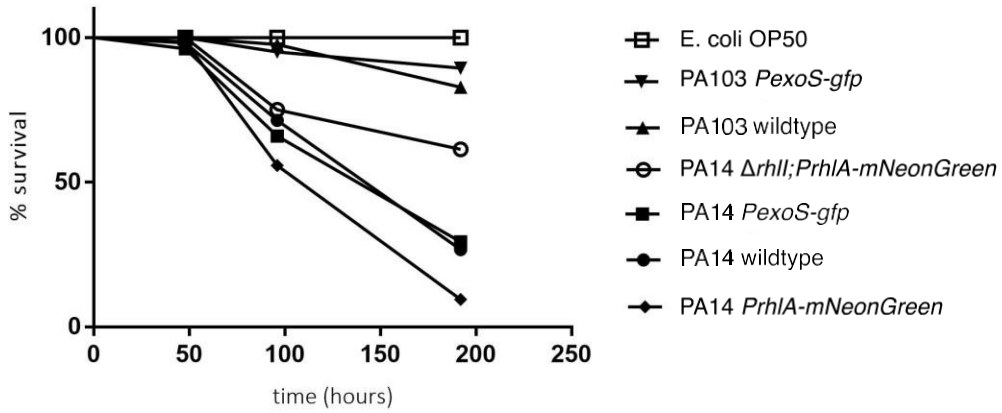
Fig. 4.3.1. Infection of C. elegans with P. aeruginosa strains*. A) Infection of C. elegans from egg-lay with PA strains: showing % survival of C. elegans after eggs were laid onto OP50 (control), PA01 and PA14 bacterial lawns. Timepoints indicate day 3, 6 and 8 from the day eggs had been laid. Error bars represent standard deviation of three replicates, statistical significance refers to comparison with E. Coli OP50. B) Infection of C. elegas from L4 with PA strains: showing %survival of C. elegans after L4 were laid onto OP50 (control), PA01 and PA14 bacterial lawns. Timepoints indicate day3, 6 and 8 from day 1 of adulthood. Error bars represent standard deviation of three replicates, statistical significance refers to comparison with E. Coli OP50.* (Unpaired t-test, *p <0.05, **p <0.01)

4.3.2. *C. elegans* infection with biofilm-competent vs biofilm-incompetent strains

The reporters pExoS and prhlA were used to perform an infection assay in a *C. elegans* model. The non-pathogenic strain *E. coli* OP50 was used as a control. Animal were initially grown onto OP50 lawns and, once Larval stage L4 was reached, these were transferred onto Pseudomonas lawns and monitored up until 200 hours. As shown in fig 4.3.2, worms fed with PA103 and PA103 *PexoS*-

gfp showed levels of survival very close to the control (90% and 95% survival respectively, compared to 100% survival in OP50), as opposed to animals fed with PA14 strains: only 25% of the animals grown onto PA14 wild type were still alive by 200 hours, very similarly to PA14 *PexoS-gfp* (~25% survival) and PA14 *PrhlA-mNeonGreen* (~10% survival). On the contrary, PA14 *ΔrhlR PrhlA-mNeonGreen* appeared significantly less pathogenic to *C. elegans*, 50% of which were still alive, at 200 hours.

Noticeably, animals fed with PA14 *ΔrhlR PrhlA- mNeonGreen* had significantly better survival percentage, compared to the non-mutant strain, which might be related to the strain forming less biofilm and therefore being less virulent; this highlights the involvement of the gene *rhlR* in biofilm formation and correlates well with the biofilm assay and fluorescence assay, described in chapter 4.2.



Statistical analysis		
condition	**p-value**	**Sig different?**
PA103 wt	0,5724	no
PA103 PexoS-gfp	0,5505	no
PA14 wt	0,0550	no
PA14 *PexoS-gfp*	0,0418	yes
PA14 *ΔrhlR PrhlA-mNeonGreen*	0,1504	no
PA14 *PrhlA-mNeonGreen*	0,0078	yes

Fig. 4.3.2 Infection of *C. elegans* with *P. aeruginosa* strains. *Survival assay was performed by monitoring survival for up to 200 hours. Animals were infected with two biofilm-incompetent strains (PA103 and PA103 PexoS-gfp) and 4 biofilm-competernt strains (PA14 wild type, PA14 PexoS-gfp, PA14 PrhlA-mNeonGreen, PA14 ΔrhlR PrhlA-mNeonGreen). Statistical analysis was carried out using Log-rank test between E. coli OP50 survival curve and survival curves of all the other conditions.*

4.3.3 The *ΔrhlR* mutation improves health profiles of PA14-infected *C. elegans*

Health profiles of *C. elegans* were further evaluated using Magnitude Biosciences' automated high-throughput imaging platforms. Movement of the worms is tracked, upon infection with the strains of interest: PA14 *PrhlA-mNeonGreen* and PA14 *ΔrhlR PrhlA-mNeonGreen.* Compared to the control OP50, movement was severely reduced by the strains PA14 wild type and PA14 *PrhlA-mNeonGreen,* while animals cultivated onto the mutant appeared to be more mobile, confirming the hypothesis that biofilm-competent strains exert a more detrimental effect compared to biofilm-incompetent ones. (47)

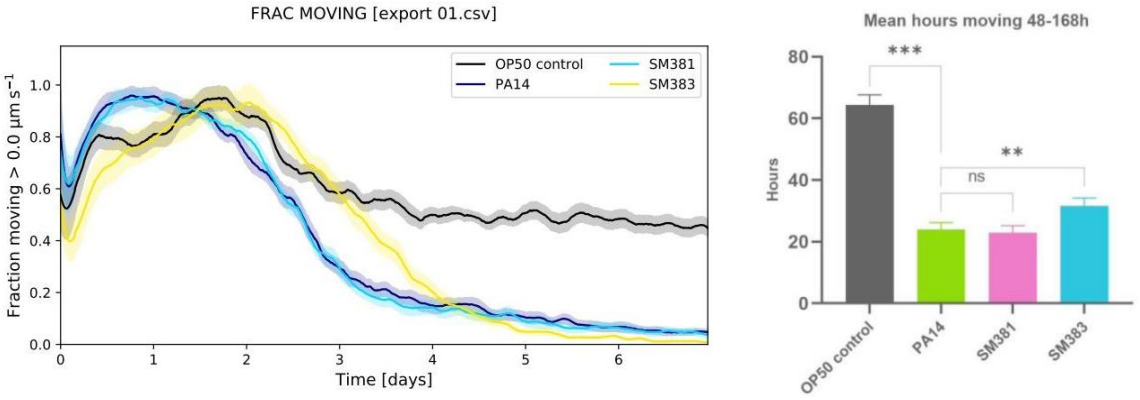


Fig. 4.3.3. C. elegans healthspan after infection with Pseudomonas strains. *Motility of the worms was monitored for 7 days starting at Larval stage (L4). Compared to cultivation onto OP50, PA14 and PA14 PrhlA-mNeonGreen (SM381) led to a significant decrease of movement of the animals. Animals grown onto ΔrhlR PrhlA-mNeonGreen (SM383) we more mobile. A difference of less than 1.64 standard errors is marked as not significant (ns). A difference between 1.64 and 2.33 standard errors is marked as one star (*), corresponding to P<0.05 on a one-sided test. A difference between 2.33 and 2.83 standard errors is marked as two stars (**), corresponding to P<0.01 on a one-sided test. A difference greater than 2.83 standard errors is marked as three stars (***) corresponding to P<0.002 on a one-sided test.*

4.3.4 In vivo imaging of *Pseudomonas* reporter strains in *C. elegans*

We wanted to see whether Pseudomonas reporter strains expressed fluorescence in vivo. To do so, *C. elegans* was cultivated with the reporter strains of interest, starting at L4 stage, and fluorescence monitored throughout adulthood, by employment of epifluorescence imaging. This allowed visualization of the animal intestine, without dissection. The intestine is in fact the primary point of entry for the bacteria into the worm and primary point of infection.

4.3.4.1. In vivo imaging of C. elegans infected with pLac::gfp reporter strains

As shown in fig 4.3.4.1, green autofluorescence was detected in the animals infected with PAO1 mutants, coming from the intestine gut granules. Bacterial fluorescence was not detected in the lumen gut, therefore GFP is not expressed by the bacteria, while infecting the worms.

These results highlight the unsuitability of these reporter strains for in-vivo visualization of fluorescence and biofilm detection in *C. elegans*.

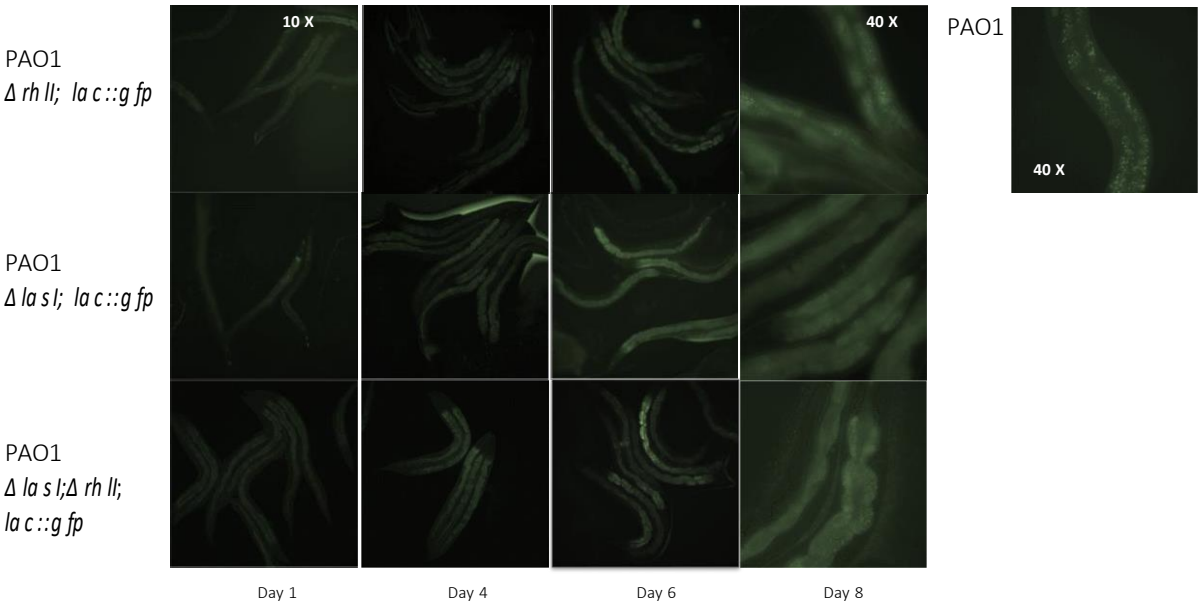


Fig 4.3.4.1. P. Aeruginosa pLac::gfp reporters do not express fluorescence in vivo in C.elegans. *Animals were immobilised and imaged using Leica DMR epifluorescence microscope. Images were taken at day 1, 4, 6 and 8 of adulthood at 10X and 40X magnification.*

4.3.4.2. In vivo imaging of C. elegans infected with *PrhlA-mNeonGreen* reporter strains

As shown in fig. 4.3.4.2, an intense fluorescence signal was detected within the intestinal lumen of the worms infected with PA14 *PrhlA-mNeonGreen.* This was particularly strong at day 5 and remained intense throughout day 7. As compared to the PA01 strains, the fluorescence had a different pattern and was not localized to the gut granules only. The lumen appeared distended and filled up with fluorescent bacteria, particularly at day 5 and 7 of adulthood. A different outcome was observed in the infection with the mutant strain, where the lumen appeared clear of fluorescence and only autofluorescence was detected. This suggested that reduced quorum sensing through the RhlI/R pathway also reduced infection in *C. elegans*.

Overall, *PrhlA-mNeonGreen* reporter strains generated good results for in vivo imaging of C. elegans intestinal bacterial infection; a major difference existed between fluorescence levels of the mutant and non-mutant strain, likely showing suitability for biofilm in-vivo studies, even though a more in depth analysis should be carried out to verify the presence of a biofilm matrix.

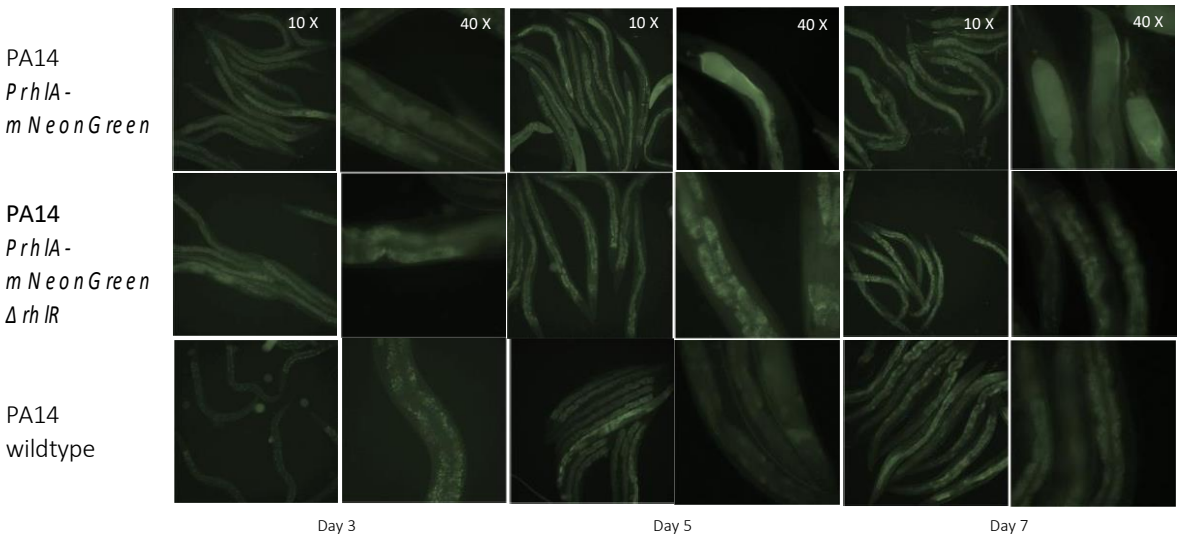


Fig 4.3.4.2. P. Aeruginosa PrhlA-mNeonGreen reporter strains express fluorescence in vivo in C.elegans. *Animals were immobilised and imaged using Leica DMR epifluorescence microscope. Images were taken at day 3, 5, and 7 of adulthood at 10X and 40X magnification.A strong fluorescent signal was detected, particularly at day 5 and 7.*

4.3.4.3. In vivo imaging of *C. elegans* infected with PexoS::gfp reporters

As shown in fig 4.3.4.3, fluorescence was also detected in the intestine of animals infected with PA14 *Pexos::gfp.* Again, the intestinal lumen of the worms appeared distended and full of fluorescence, suggesting bacterial colonization. On the other hand, no signal was detected in PA103 *Pexos::gfp*, hinting an inability of the strain to form biofilm and/or to colonise *C. elegans.* Fluorescence started to be visible at day 5 of adulthood and increased noticeably at day 7.

PA14 *Pexos::gfp* showed potential suitability as biofilm fluorescent marker, as indicated by the intense fluorescence signal coming from the animal gut, therefore it would be suited for future studies on in vivo biofilm -detection methods.

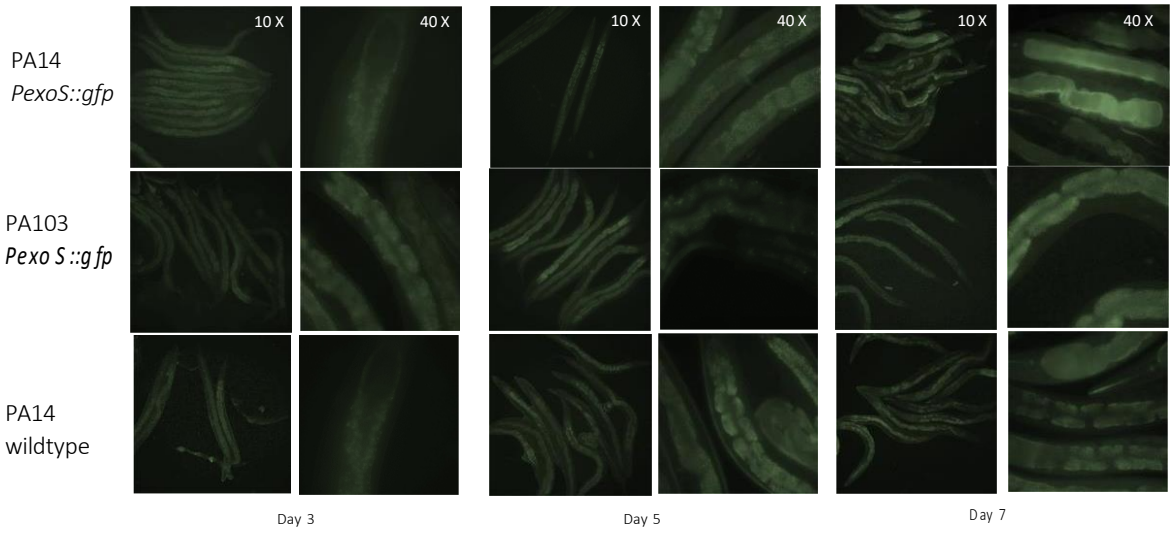


Fig 4.3.4.3. P. Aeruginosa Pexos::gfp reporter strains express fluorescence in vivo in C.elegans. *Animals were immobilised and imaged using Leica DMR epifluorescence microscope. Images were taken at day 3, 5, and 7 of adulthood at 10X and 40X magnification. A strong fluorescent signal was detected, particularly at day 5 and 7.*

4.4 Using biofilm inhibitors

4.4.1 Biofilm inhibitors reduce biofilm in vitro

The compound 4-Nitropyridine-N-oxide (NPO) is a well know biofilm inhibitor in *P. aeruginosa* that we utilized to confirm the development of biofilm by the pRhlA *Pseudomonas* reporter strains. Three different concentrations of 0.1 mM, 1mM and 10 mM, were mixed with cultures of pRhlA-mNeonGreen and let grow onto a microtiter plate, to measure Fluorescence intensity of the formed biofilm. As shown in the graph underneath (fig. 4.4.1), fluorescence intensity of the reporters progressively decreased by increasing concentrations of NPO.

Due to the well-known action of the NPO molecule as biofilm inhibitor, the decreased fluorescence expression from cultures of pRhlA- mNeonGreen, upon increasing concentrations of NPO, confirmed the correlation between fluorescence expression and biofilm production by the reporter strain and the suitability of the fluorescence assay as a readout of bacterial biofilm levels.

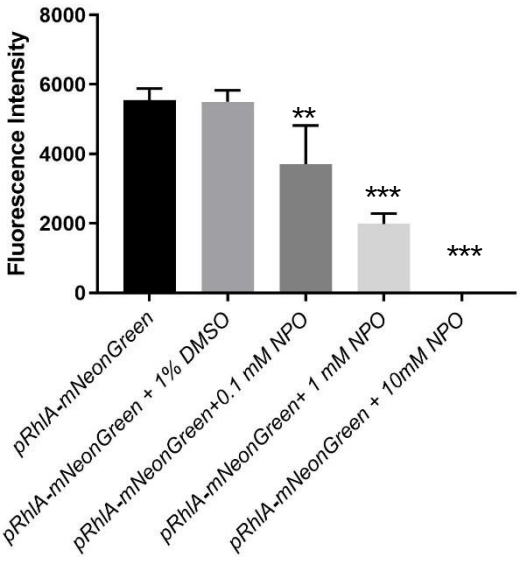


Fig. 4.4.1 Effect of a biofilm inhibitor onto biofilm formation in P. aeruginosa pRhlA reporter strains. *GFP fluorescence was measured in microtiter plates to quantify biofilm formation, after culture exposure to a biofilm inhibitor molecule NPO. Three biological repeats were performed for each strain. Error bars represent standard deviation of the repeats, statistical significance refers to comparison with pRhlA-mNeonGreen without NPO (*One-way ANOVA, *p <0.05, **p <0.01, ***p <0.0001). *Panels showing: pRhlA-mNeonGreen, pRhlA-mNeonGreen + 1% DMSO, pRhlA-mNeonGreen+0.1 mM NPO, pRhlA-mNeonGreen+ 1 mM NPO, pRhlA-mNeonGreen + 10mM NPO.*

4.4.2 Health profiles of C. *elegans* do not improve with biofilm inhibitors

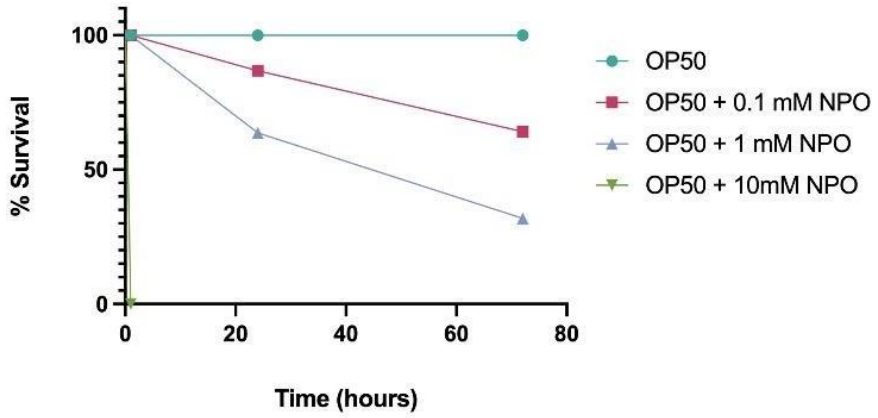
Next, we evaluated the use of NPO for in-vivo study in *C. elegans*, to see whether the compound improved health profiles of the worms, thanks to its anti-biofilm properties.

Unfortunately, the use of NPO for in vivo studies proved to be challenging, likely due to the compound exerting a negative effect onto *C. elegans*. As a preliminary test, worms were first cultured in liquid with the non-pathogenic strain *E. coli* OP50, treated with NPO concentrations of 0.1 mM, 1mM and 10mM as for the in vitro assay. As shown in fig. 4.4.2 A, when treated with concentrations of 10mM, worms died within an hour, therefore we excluded this concentration for in-vivo studies. Overall, compared to the OP50 control without NPO, 0.1 mM NPO concentrations appeared to be tolerated, with about 70% of the worms being alive at day 3, compared to those treated with 1mM NPO concentrations, where only less than 50% of worms were alive by day 3. Despite noticing a slight negative effect on worm's survival rates, despite being cultured with a non-pathogenic strain OP50, we explored the effect of NPO onto PA strains, to see if the compound made PA strains less pathogenic to the worms.

C. elegans was cultivated in liquid, with bacterial cultures of *E. coli* OP50 and PA14, treated with concentrations of NPO ranging 0.1-1 mM. Higher concentrations were excluded from the trial, due to high mortality rates of the worms as soon as exposed to the solution, as shown in fig. 4.4.2 B Untreated OP50 and PA14 cultures were used as controls.

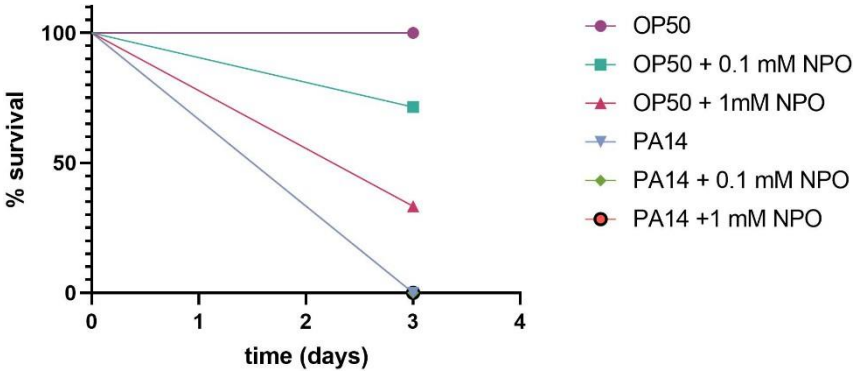
As expected, animals grown on *E. coli OP50* did not die during the 3-days timeframe. On the contrary, *C. elegans* health profiles significantly worsened when exposed to concentrations of NPO, despite OP50 not being pathogenic to the animal: only 60 % and 20% of the worms were still alive after 3 days, when grown into 0.1 and 1mM NPO and OP50 solutions respectively. A more dramatic effect was observed in worms cultivated in PA14, despite treatment with NPO: no worms were alive after 3 days, for all the conditions (PA14 only, PA14+ 0.1mM NPO and PA14+ 1mM NPO).

A



Statistical analysis		
condition	**p-value**	**Sig different?**
OP50 + 0.1 mM NPO	0,0001	yes
OP50 + 1 mM NPO	<0,0001	yes
OP50 + 10 mM NPO	<0,0001	yes

B



Statistical analysis		
condition	**p-value**	**Sig different?**
OP50 + 0.1 mM NPO	<0,0001	yes
OP50 + 1 mM NPO	<0,0001	yes
PA14	<0,0001	Yes
PA14 +0.1 mM NPO	<0,0001	Yes
PA14 + 1 mM NPO	<0,0001	yes

Fig. 4.4.2. Survival assay of C. elegans using a biofilm inhibitor molecule. *A) Worms were exposed to cultures of OP50 treated with NPO concentrations ranging 0.1-10mM. Worms exposed to 10mM concentrations died within 1 hour; less than 50% of the worms cultivated with 1 mM NPO were still alive after 3 days; more than 50% of the worms cultivated with 0.1 mM NPO were still alive after 3 days. Statistical analysis was carried out using Log-rank test between E. coli OP50 survival curve and survival curves of all the other conditions. B) Worms were exposed to cultures of OP50 and PA14, treated with the biofilm-inhibitor molecule NPO, in concentrations ranging 0.1-1 mM. Despite observing in vitro lower biofilm levels in biofilm-competent bacteria after treatment with NPO, the molecule did not improve health profiles of the animal. Instead, it worsened survival rates. Statistical analysis was carried out using Log-rank test between E. coli OP50 survival curve and survival curves of all the other conditions.*

CHAPTER 5: DISCUSSION

The project initially focused on the creation of an in-vivo model for biofilm detection system in *C. elegans*. The aim was to use a molecular biology approach to create a transgenic *P. aeruginosa* carrying a biofilm-specific fluorescent reporter and to support the development of an assay, available to industry and academic researchers. The next part of the project focused on evaluating if *Pseudomonas* fluorescent reporter strains generated by other labs are useful for bacterial biofilm detection.

5.1 The current lack of in vivo models for biofilm studies.

Bacterial biofilms are complex structures, characterised by bacterial colonies adherent to surfaces (either animal tissues or abiotic surfaces, such as medical implants) and characterised by a complex structure, made of polysaccharides, rhamnolipids and eDNA, offering nutrients and protection to the bacteria. (48) Particularly due to their acquired antimicrobial tolerance, biofilm-associated infections are difficult to treat and contribute to many chronic infections, representing a serious global health. (48)

Unfortunately, most industrial and diagnostics tests to study their presence and characteristics, are in vitro testing, which do not mimic well enough in vivo conditions and therefore lack translatability. (49) In vitro models include static models such as microtiter plate assays, agar plates and Calgary biofilm device, while dynamic models include flow cell systems and biofilm reactors.(50) Dynamic models appear more accurate, due to exposure of the biofilm to a stressor such as a constant flow of medium over the biofilm, mimicking an in vivo host environment.(50) However, new assays need to be developed to represent more accurately the interaction between the host immune system and biofilms and providing a readout of the host's health. In fact, by better understanding the mechanisms of biofilm and host interactions, it will be easier to develop new treatments and eradicate them. (51) Murine models have been used to study biofilm formation in vivo, such as described by Attaran et al. for the study of *H. pylori* biofilms in the host's

stomach, but these are far from being employed in industrial/diagnostics settings, due to limitations in experimental designs, often lengthy and costly. (52)

To fill this market gap, we suggest the employment of the nematode *C. elegans* for in-vivo research on bacterial biofilms, upon employment of biofilm-specific fluorescent markers.

5.2 *C. elegans* for in vivo biofilm studies

C. elegans is a transparent organism which allows easy detection of fluorescent markers through fluorescent microscopy (53). The animal transparency also allows observations of cell physiology and changes in response to the biofilm infection. (53). Based on this rationale, we tried to develop a biofilm specific marker for real time observation and quantification of bacterial biofilm in the living organism *C. elegans*, through fluorescence quantification. Although *C. elegans* is suitable as a model organism for such approaches, the nematode has never been used for the purpose of biofilm monitoring before.

The use of this model for research has several advantages: on top of the animal transparency*, C. elegans* can be cultured in large synchronic populations and their development happen in a very short time; (54) the small dimensions of the animal make it useful for high-throughput screening approaches; (54) the animal is also very easy to maintain and can be easily frozen and revived.

(54) Finally, *C. elegans* has a fully sequenced genome which comprises about 65% of human disease-related genes, making it a perfect organism to study human disease. (54) Therefore, compared to rodent and other animal models, a *C. elegans* model is cost and time-effective and reduces ethical impact. (54)

5.3 Infection model of *C. elegans*

The biofilm competent and multi-drug resistant species *P. aeruginosa* has been chosen to infect *C. elegans*, since previous studies have proven its pathogenicity towards the nematode and infection models were already described. (55) *P. aeruginosa* is a gram-negative bacterium for which we are

in great need of new therapeutics, due to its resistance to antibiotics.(42) About 5000 strains of the gram-negative bacterium exist, among which PA01 and PA14 are considered the most common laboratory reference strains, exhibiting a moderate and extremely virulent phenotypes, respectively.(42)

We questioned whether either PA01 or PA14 would be most suitable for *C. elegans* infection assays, and to determine how long it would take to each strain to express its pathogenicity towards the nematode.

As expected(56) our results showed that PA14 established a much virulent phenotype on the worms, which died quickly, within 8 days of infection, while worms infected with PA01 seemed to survive for longer than 8 days, without any sign of reduced health (such as locomotion impairments), similarly to our control (Fig.4.3.1).

Our results also showed that infection varied, depending on the stage animals were infected at: those which were initially grown onto OP50 and infected with *Pseudomonas* at L4 stage survived at higher percentages, as opposed to those fed with *Pseudomonas* strains since eggs.

For the purpose of creating a biofilm assay, we wanted to be able to collect observations on the worms' health for at least 7-8 days. Therefore, it was concluded that PA14 would allow better simulation of a biofilm infection in *C. elegans*, but that worms would be infected at L4 stage. This would prevent an overly pronounced loss of worms, which we speculate is due to worms during development (from larval stage L1 to L4) being more susceptible to infection, therefore dying early or not developing into adulthood, perhaps due to avoidance of food.

5.4 Exploiting QS in *P. aeruginosa* to build a color-based assay

Research has shown that quorum sensing (QS) plays a major role in *Pseudomonas aeruginosa* biofilm formation (56). This process is under tight genetic regulation, with three main QS circuits regulating multiple sets of genes or operons: the las, rhl and pqs QS systems. These systems rely on molecules, known as QS autoinducers; acylated homoserine lactone (AHL) are common QS

autoinducers in gram-negatives bacteria, such as *Pseudomonas aeruginosa.*(57) QS systems usually rely on AHL synthase proteins, normally a LuxI homolog, to produce autoinducer molecules; transcriptional activators, normally LuxR homologues, bind their partner synthase proteins, forming LuxR-AHL complexes which regulate gene expression eliciting a group behaviour, such as biofilm formation.(57) In *Pseudomonas aeruginosa*, the LuxI/R pairs consist of lasI/R and RhlI/R; LasI produces the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), which exerts its effect on *LasR* and also inducing *rhlR* transcription;(57) RhlI synthase produces the autoinducer *N*-butanoyl-L-homoserine lactone (C4-HSL), which binds RhlR, activating genes encoding for virulence and other biofilm components, such as rhamnolipids.(57)

In our study, we initially focused on *rhlA, rhlB and rhlI,* for their link with rhamnolipid production (28), necessary for biofilm virulence and development (27), and *pqsA* and *pqsB* for their link with eDNA release, a major element of biofilm (25). The *rhlAB* operon encodes for a rhamnosyltransferase, which is necessary for rhamnolipid production.(58) As shown by Kievit et al. (10), rhamnolipid was in fact reduced in delta rhlI mutant, and abolished in double delta l*asR:rhlR* mutants(58). Rhamnolipids are biosurfactant (surface-active molecules) which presence is necessary to maintain open channel structures, necessary for the maintenance of the biofilm architecture during late stages of biofilm development, where the bacteria communicate very actively (59). This might be due to the impairment of cell-cell interactions and attachment of bacterial cells to surfaces.(60) eDNA is also a crucial component of biofilm, particularly in relation to the biofilm matrix formation(61). Often found in lung mucosa of cystic fibrosis patients, eDNA makes the biofilm more stable, by mediating molecular interactions within the biofilm, and also has a role in biofilm drug resistance, due to its acidifying properties.(62) The PQS system (Pseudomonas quinolone system) is characterised by the pqsABCDE cluster; it has been shown (25) that biofilms of pqsA mutants contained less eDNA, compared to the wild type. A link has also been shown with glycolipids, virulence factors and membrane vesicles production. (25)

With the assumption that *Pseudomonas aeruginosa* biofilm requires the expression of the above-described genes, we attempted to create transgenic bacteria whose biofilm production could be monitored and quantified by expression of a biofilm-specific fluorescent reporter. The employment of fluorescent proteins, such as Green Fluorescent proteins (GFP), is of great benefit to scientific research, particularly to understand gene expression; these can be modified utilizing molecular biology techniques, to generate fusion proteins which express customised shades of fluorescence. (14) Typically, the gene of interest is linked to the gene encoding for the fluorescent reporter, so that when the first is switched on, the fluorescent protein will also be expressed. (63) Examples include ratiometric reporters, which exhibit non disruptive changes in fluorescence, in response to changes in a parameter of interest. (14)(64) Therefore, physiological variations in the concentration of the parameter of interest, will result in measurable and reproducible variations in the excitation and emission spectra of the reporter. (14)

For our assay, the employment of a constitutively expressed GFP and biofilm-selective mCherry would be convenient to distinguish between planktonic cells (characterised by green fluorescent expression) and biofilm (characterised by orange fluorescence, derived by the mix of red and green fluorescence). Despite fluorescent transcriptional reporters being extensively used as biomarkers, in-vivo applications of this type are still limited, particularly due to reduced signal intensity.

5.5 Unsuccessful cloning: possible causes

Unfortunately, as explained in section 3.2-3.5, we were not successful with the cloning, due to a problem with our vector, which was not digested as expected. This was shown by the unexpected bands obtained in the agarose gel performed to check the quality of the plasmid (fig. 3.1.1.C) and subsequent gels. Undigested plasmids normally yields two or more bands in their gel lane, due to their non-linearity; these bands represent the secondary structure of the plasmid, which often appear in two forms, a supercoiled circular DNA which migrates faster and further down in the gel and an open circular form, which moves slower and therefore appear higher in the gel. Unfortunately, the same bands persisted in most of the plasmid digests (from lane 3-6), showing

a lot of residual undigested plasmid and poor digestion. The presence of these bands demonstrating that, unfortunately, there was a lot of residual undigested plasmid and poor digestion throughout the whole cloning process.

Nevertheless, the presence of colonies in our ligation positive control sample confirmed that there likely was not a problem with the cloning protocol carried out (fig. 3.1.5, B). Different causes may lead to colonies which do not contain the plasmid of interest, such as 1) recombination of the plasmid might have occurred, 2) incorrect PCR amplicon might have been used, 3) the DNA fragment could have been toxic to the cells, 4) mutations might have occurred within the sequence. (65) We tried to adjust the protocol to low-copy number miniprep, but unfortunately the issue was not solved.

Due to lack of time, we were not able to troubleshoot any further, and we decided to evaluate the use of reporter strains already existent, found by looking in the literature.

5.6 Using *P. aeruginosa* reporter strains to detect biofilm in vitro

We focused on *P. aeruginosa* reporter strains which were already described in the literature, and we tested their use in our *C. elegans* infection model. The overall aim was to determine their application into biofilm studies. Seven transgenic strains were obtained, expressing fluorophores, related to QS signalling processes: PDO-100, PAO-JP1 and PAO-JP2 obtained from Gary Robinson (University of Kent) constitutively expressing GFP in PAO1 background under a lac promoter (P*lac::GFP*), hosting mutations in the *las* and *rhl* QS system (a single *lasI* knockdown, a single *rhlI* knockdown, a double knockdown in *lasI* and *rhlI* respectively); (56) SM381 and SM383 obtained from Bassler lab, (Princeton University) hosting a mNeonGreen transcriptional reporter fusion to the *rhlA* promoter (P*rhlA::mNeonGreen*), in a PA14 background, the second strain also hosting a *rhlR* knockdown. (57) Two more strains were obtained from the Hall lab (University of Kent) harbouring a GFP reporter fusion to the exoS promoter (*PexoS::GFP*) in PA14 or PA103 background. (46) These are linked to the secretory pathway III, which has been shown to contribute towards biofilm formation. (45) We initially tested in vitro ability of the strains to form biofilm; this assay, which is carried out in a microtiter plate, relies on crystal violet staining of the bacterial wall peptidoglycans; biofilm quantification is provided by absorbance measurements in the plate reader.

Our results show that, in comparison to a biofilm-incompetent strain (*E.coli* BL21), PA01 and PA14 had increased ability to form biofilm in vitro, as opposed to PA103 which had reduced ability. As expected, decreased ability to form biofilms was also noticed in the *ΔlasI;ΔrhlI* mutant; however, ability to form biofilm did not seem impaired in the single mutants *ΔrhlI* and *ΔlasI,* particularly the first appeared to form consistent levels of biofilms. These results suggest that single mutations are likely insufficient to have an impact on biofilm formation, unless coupled to other mutations, such as in the case of the double mutant *ΔlasI;ΔrhlI.* Interestingly, *rhlR* appeared to have a more crucial role in biofilm formation, as observed in the biofilm profile of the *ΔrhlR* knockdown: compared to wild type, this showed two-fold less levels of biofilms, suggesting the important role of the rhl system in biofilm formation in *Pseudomonas aeruginosa*.

Other studies have in fact studied the role of the rhlR quorum sensing receptor control in biofilm development of *P. aeruginosa*, showing reduced virulence and production of defective biofilms (thin and undifferentiated) in strains harboring QS mutations. (66) Lot of attention has been paid to the LasI/R system, due to its location at the start of the QS signal transduction cascade, however, RhlR has been shown to be the primary QS regulator during host infection, in a Drosophila model.(67) In fact, it has been noticed by Limmer at al that *lasR* loss of functions often arise in cystic fibrosis infections, highlighting the minor role of the las QS system in chronic infections linked to biofilm formation, compared to the rhl system.(67)

Thanks to the biofilm assay we had a clear idea of each strain's potential to form biofilm, so we next employed a fluorescence-based assay to evaluate the fluorescent signals emitted by each strain, when forming a biofilm. The same microtiter assay protocol was followed, whereby cultures are grown onto a solid surface but, instead of staining them with crystal violet, after having washed planktonic cells off, we measured the fluorescence emitted by the biofilm, using a plate reader. This approach gave results which were partly consistent with the biofilm assay: the double mutant *ΔlasI;ΔrhlI* expressed lower levels of green fluorescence, compared to the single gene knockout *ΔrhlI and ΔlasI,* the first of which expressed the highest levels of fluorescence intensity. As

expected, the *ΔrhlR* mutant also expressed 3.5 lower levels of fluorescence, compared to the strain hosting *PrhlA-mNeonGreen*. Finally, high levels of fluorescence intensity were observed in the pJNE05 reporter strain, with high levels expressed in both PA103 and PA14 background, therefore contradicting the crystal violet assay, where PA14 background seemed to yield higher levels of biofilm, compared to PA103.

Overall, the lac:gfp reporter strains had up to 6-fold higher levels of fluorescence, compared to the other reporter strains. (Fig.4.2.2 A) However, as indicated by the biofilm assay, fluorescence cannot be correlated with biofilm expression, since the lac promoter is not biofilm specific. Nevertheless, the mutant strains were very helpful to observe the impact on biofilm formation exerted by each QS system. On the other hand, both rhlA and Exos seem more suitable for biofilm studies, due to the rhl and Type III secretion system involvement in biofilm expression. Nevertheless, further optimisation should be carried out, for the exact correlation of fluorescence with biofilm formation. Finally, fluorescence was also detected in PA14 wild type, highlighting the autofluorescence problem which could be improved by using a different colour of fluorescence, as initially planned in this study.

5.7 Using *C. elegans* to study biofilm pathogenicity in vivo

Once established the reporter strains' ability to form biofilms and fluorescence emission, we evaluated their use in vivo. This was done by cultivating the invertebrate *C. elegans* with the Pseudomonas reporters strains, along with *E. coli* control strains. Animals used had reached larval stage L4, which we had previously proven to be more convenient rather than using worms eggs. Fluorescence emitted by the bacterial strains from within the worm's intestine, was monitored by using epifluorescence imaging, throughout the worm's adulthood. Pathogenic bacteria normally infect *C. elegans* through the gut, which is their first entry point into the animal, though their mouth opening. (68)

As expected, when using PA14, PAO1 and PA103 wild types, no bacterial fluorescence was detected, apart from the green autofluorescence coming from the gut granules, which was expected. The same was observed for the plac::gfp expressing reporter strains. Despite having observed in vitro fluorescence expression and biofilm formation in those strains, particularly in the *ΔrhlI and ΔlasI* mutants, this might suggest that the fluorescence reporter was not also expressed in vivo.

A different trend was observed in animals treated with PA14 carrying pexos:gfp: strong fluorescence was detected within the intestinal lumen of the animals; intestines were also swollen and distended, suggesting bacterial colonisation and infection. As expected, PA103 background did not show the same result. The laboratory strain PA103 is in fact known for its aflagellate phenotype, resulting from a single amino acid change in the flagellar regulator FleQ; (69) are important for bacterial swimming motility and biofilm development in *P. aeruginosa* and this might have had an impact on biofilm formation in the *C. elegans* intestine. ((70)

Strong fluorescence was finally detected from the *prhlA*-mneongreen reporter strain in PA14 background: the intestinal lumen looked distended and filled up by fluorescent bacteria, probably indicating biofilm presence. Contrarily, the mutant *ΔrhlR prhlA-mNeonGreen* did not express any fluorescence, suggesting that reduced *rhl* quorum sensing signal affect biofilm formation, making the bacteria less capable of colonising the worm intestine.

5.8 Biofilm incompetent reporter strains have reduced pathogenicity

Next, we performed infection assays to evaluate virulence of the reporter strains ExoS and rhlA in *C. elegans.* The nematode was initially cultivated onto OP50 and transferred to PA strains once they reached larval stage (L4). Survival rates were monitored at intervals, up until 200 hours. Compared to an OP50 control, where animals did not die throughout the whole experiment, animals cultivated with PA14 strains showed the highest death rate, particularly PA14 wt, PA14

PexoS::GFP and PA14 prhlA-mNeonGreen, where about 40%, 30% and 20% respectively of the animals have survived by the end of the assay. Health profiles were improved in the mutant *ΔrhlR,* and in PA103 strains, suggesting that *P. aeruginosa* infection in C. elegans involves biofilm formation and that quorum signalling through rhlR has a role in Pseudomonas infection. These results were further confirmed by measuring health profiles of C*. elegans,* using Magnitude Biosciences automated high-throughput imaging platforms: compared to the control, movement was severely impaired in nematodes infected with PA14 wild type and *PrhlA-mNeonGreen* reporter strain*,* while the mutant *ΔrhlR* had a lower impact on the animal locomotion.

5.9 The effect of biofilm inhibitors on *prhlA::mNeonGreen* fluorescence expression

Finally, to confirm the presence of biofilm within C*. elegans* we employed a known biofilm inhibitor, 4-nitropyridine-N-oxide (NPO). The compound NPO is an antiadhesive molecule which gets absorbed by surfaces or bacteria, resulting in physical and chemical changes which compromise bacterial adhesion, before the bacteria can activate quorum sensing mechanisms and establish a biofilm. (71) Rasmussen et al. showed that biofilm is reduced when using the compound at a concentration of 1mM. (71) carried out an in vitro assay for fluorescence detection, using the *prhlA::mNeonGreen* reporter strain, cultured with three different concentrations of NPO ranging 0.1 mM-10mM. When comparing with a biofilm incompetent bacterium, which did not express any fluorescence, a strong signal was detected from the *rhlA::mNeonGreen* reporter strain culture in the absence of NPO, suggesting that biofilm was being produced and the bacteria were expressing the fluorescent reporter; this steadily decreased with increasing concentrations of NPO, suggesting that the compound compromised the ability of the bacteria to form a biofilm, therefore also compromising the expression of fluorescence in vitro. These results further confirmed that a fluorescence-based assay can be used in correlation to biofilm production.

5.10 The effect of biofilm inhibitors on a *C. elegans* biofilm infection model

Unfortunately, the employment of NPO in vivo resulted more challenging. Animals were exposed from L4 stage to PA14 wild type cultures, which we previously proved to be a biofilm competent

strain, treated with NPO concentrations (0.1mM and 1mM), and their survival rates were recorded at intervals. We excluded 10mM concentrations, due to sudden death of the nematode, within 1 hour of exposure to the compound. (Fig. 3.15 A) OP50 was used as a control and treated with concentrations of NPO too. Despite NPO reducing biofilm levels in *P. aeruginosa*, we did not report any improvement in the nematode health. Apart from our control (*C. elegans* cultured with OP50 without NPO), where the worms did not die throughout the whole assay, all the worms treated with PA14 died within 3 days, and only 60% and 25% of those cultured with OP50 with NPO concentrations (0.1mM and 1mM respectively) survived. These results indicated that NPO is not tolerated by the worms, perhaps due to a toxic effect of the compound., despite reducing bacterial biofilms. The chemical is in fact considered toxic and irritant to humans, with some evidence of carcinogenic effect, and the same might be exerted on the nematode. ((72)

Despite inconclusive in regard to our biofilm study, these results showed that *C.elegans* could be helpful in compound screening, by providing early warnings of safety issues, for instance during the preclinical research phase of drug development. In fact, regulations entail that two mammalian species are used during this phase, to test drugs' safety. *C. elegans* might be employed in preliminary screenings, to exclude drugs with signs of toxicity, before proceeding with more time-consuming and expensive models, such as murine models. Contrarily to in vitro studies, *C. elegans* represents a whole organism model which could reveal developmental and reproductive toxicity and health damage from prolonged chronic exposure to the compound, in just one experiment. (31)

Many QS-inhibitory compounds have been identified, of both natural and synthetic origin (71); particularly, studies have shown the potential of certain compounds at reducing virulence in *P. aeruginosa* by improving bacterial clearance and reduce mortality; (73) some of these include garlic-derived molecules, which impaired biofilm formation both in vitro and in vivo models, thanks to Quorum sensing inhibition activity, particularly the lasI/lasR QS system and rhlI/RhlR systems, as reported by Persson et al. (73)

Therefore, other options, such as garlic extract compound, should be explored for *C. elegans* in vivo biofilm inhibition models.

5.11 Strengths and weaknesses

Overall, our infection assay has proven to be effective for the study of biofilm-competent pathogenic bacteria. The infection assay is a simple experiment, which requires inexpensive reagents and basic equipment, often found in laboratories, such as a dissecting microscope and microplate reader. The use of sterile *glp-1* worms avoided the need of transferring worms to distinguish them from the progeny, therefore it made the experiment simple and hassle-free, ideal for testing different conditions. However, *glp-1* worms might differ from the canonical wild type strain

C. elegans N2 and results might also be affected. In fact, *glp-1* mutations cause germ cells to prematurely enter meiosis, with consequent depletion of proliferative cells and sterility upon temperature shifts (74); Alper et al. findings suggest that innate immune response of *C. elegans* to gram-negative bacteria is controlled by the germline, making the nematodes more resistant to the bacteria *Pseudomonas aeruginosa.* (75)

In vitro techniques, such as the biofilm assay, showed suitability for the detection of biofilm formed by *Pseudomonas aeruginosa* strains. The results obtained were consistent for each condition and repeated by another member of the Ezcurra lab, who found similar results. However, CV staining for biofilm detection has some limitations. In this process, negatively charged components of biofilms, such as polysaccharides, bind to the positively charged CV stain. An increase in negatively charged components released by dead cells and trapped by the biofilm matrix may increase the formation of CV complexes, leading to erroneous interpretation of biofilm presence. Moreover, variations in technical protocols often lead to variable results (such as time of bacterial growth, starting CFU/ml and medium used), therefore these techniques should be used taking into account the basis of crystal violet interactions and different repeats should always be carried out. (76)

Real time imaging of transgenic bacteria in *C. elegans* allowed detection of GFP green fluorescence signal, through DMR microscopy. Fluorescent microscopy is a key element in *C. elegans* research, often used to localise proteins, organelles and anatomical structures and monitor physiological parameters. (77) This type of assay is non-invasive, without requirement of dissecting the animal, thanks to the nematode transparency, therefore preserving integrity and viability of the cells. Using our epifluorescent microscope setup, we were able to yield high quality images which were available for analysis straight away. However, fluorescent proteins, which often exhibit good optical properties in vitro, may not necessarily behave in the same way, in *C. elegans*. (77) For instance, cellular pH may influence brightness and photostability of the reporter. (77) Moreover, the signal of GFP fusion proteins is masked by autofluorescence emitted by intestinal lysosome-related gut granules, making it hard to visualise weak signals; (78) autofluorescence also increases due to oxidative stress, aging and heat stress, therefore GFP filter set ups need to be optimised in order to separate the GFP signal from autofluorescence. Using red or yellow fluorescent proteins might overcome this problem. (78)

One more weakness of this study is that only 30 worms were cultivated for each condition, therefore more worms could be included to give a better picture.

5.6 **Conclusions and future research**

Overall, these results show that it is possible to establish an infection assay in *C. elegans* using *Pseudomonas* but only from L4 stage and using a liquid assay. Infecting the worms from L4 entails longer survival of the animals, allowing enough time for the bacteria to establish a biofilm infection and allowing the monitoring of the nematode's health in the meantime. A liquid assay also prevents worms' loss, which otherwise crawl off agar plates, trying to avoid pseudomonas virulent strains, probably due to the worm’s sensitivity to infectious compounds produced by the bacteria. (79)

Preventing worms’ loss means preventing censored data, making the assay more precise.

Finally, taken together, these results suggest that the *Exos* and *RhlA* reporter strains are suitable for in- vivo monitoring of biofilm formation in *C. elegans;* this is indicated by biofilm-incompetent strains being unable to express fluorescence in-vivo, as opposed to strong biofilm competent strains, which produced high fluorescence levels. Further optimisation may lead to a new diagnostic tool which exploits *C. elegans* advantages, such as transparency, short generation time and reduced maintenance costs. Unfortunately, green fluorescence is not ideal for gut screening of *C. elegans*, due to green autofluorescence coming from gut granules, and also from the bacteria *Pseudomonas*. For this reason other fluorescent proteins, such as red mCherry protein, could be employed, to be able to visualise bacterial biofilms more sharply.

Overall, *C. elegans* is good to observe bacterial colonisation happening within the worm's intestine, but further studies should be performed to confirm the presence of a biofilm. Employment of biofilm inhibitors molecules could be a good approach, even though these should be carefully selected by performing a toxicity test beforehand, so that they do not cause any harm to the worms and avoiding waste of time and resources. If, as we speculate, bacterial biofilms were the main cause of a deadly infection phenotype in *C. elegans*, upon employment of a biofilm inhibitor, worms' health span should be extended and health profiles improved. Unfortunately, the chemical NPO, which seemed a good candidate for an in-vitro approach, resulted unsuitable for in-vivo studies, since it caused the nematodes' death. Some compounds, such as garlic extract, could be employed in future studies, since this has proven to inhibit Quorum Sensing and it is also a more natural compound which might not be harmful to the nematodes, giving a clearer result. More tests could be employed, to prove the presence of biofilm within *C. elegan's* gut, such as intestine tissue isolation for fluorescent staining with DAPI for eDNA presence. (80) Exopolysachcarides present in microcolonies could also be visualised by immunostaining, such as explained by Mulcahy et al., in a *Drosophila* study for in-vivo biofilm infection where fluorescein isothiocyanate (FITC) conjugated Hippeastrum hybrid Lectin (HHA) was used. (81)

Overall, our data are the results of many repeats, confirming reliability and reproducibility. However, to further improve this assay and to obtain even more reliable data, at least 50-100 worms should be cultivated for each condition; in fact, the larger the study sample size, the smaller the margin of error, avoiding to report false-negatives or false-positive findings. Finally, the commonly used Bristol (N2) *C. elegans* strain could be used in following studies, to avoid using strains which are more resistant to *Pseudomonas* infection, such as *glp-1*, therefore yielding more trustworthy results, despite the drawback of having to transfer adults worms to fresh media, to separate from the progeny.

This project will be taken over by another student in the Ezcurra lab, focusing on de-novo transgenic reporter design, in the attempt of making biofilm-specific fluorescent tag, as initially attempted by this project. There is urgency to create in-vivo biofilm detection methods, due to the occurrence of biofilm related infections and their resistance to antibiotics. The gap in antimicrobial drug discovery is in fact alarming, particularly when looking at the shortage of effective drugs to combat biofilms. It is certain that, due to the multifactorial nature of biofilm, a combinatorial approach should be used to eradicate them. Therefore, biofilms should be studied in-vivo, in model organisms such as *C. elegans*, to reveal useful insights which are not observable in vitro; more work should be carried out to implement standardized methods so that c. elegans models are used more extensively in industry and clinical environments, fully exploiting its potential.

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