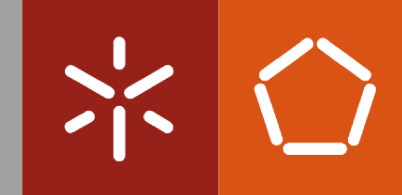




Reprogramming *Pseudomonas aeruginosa*: an in depth study on phage-host interaction

Ana Catarina Ribeiro Brandão

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Universidade do Minho
Escola de Engenharia

Arenberg Doctoral School
Faculty of Bioscience Engineering



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**Reprogramming *Pseudomonas aeruginosa*:
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Doctoral Thesis
Ph.D. in Chemical and Biological Engineering
Ph.D. in Bioscience Engineering

Work performed under supervision of
Professor Doctor Joana Azeredo
and
Professor Doctor Rob Lavigne
and
Doctor Diana Priscila Pires

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STATEMENT OF INTEGRITY

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ABSTRACT

Phages are bacterial viruses with high interest for science and technology due to their applicability in human health, biotechnology, agriculture, and veterinary, as well as in synthetic biology. As therapeutic agents, phages can be used to treat life-threatening bacterial infections, such as *Pseudomonas aeruginosa* infections, although its approval in human medicine is mainly limited by the lack of knowledge about phage mode of action under *in vivo* conditions and bacterial responses towards phage infection. In this work, RNA-seq of phage-infected bacteria grown under different media conditions and adhered to human lung epithelium was performed to capture a global view of the transcriptional events that occur under phage therapy. Thus, it was possible to prove that *P. aeruginosa* was significantly more virulent when cultured in media used to grow human lung epithelial cells, as well as when adhered to a monolayer of human lung epithelium, than when cultured in lysogeny broth. During phage infection, it was observed that global transcriptomes are substantially different at early infection, an important phase during which phage takeover of the bacterial cell occurs, with particular changes in metabolic pathways associated with energy acquisition. Curiously, in all growth conditions, there is a set of common mechanisms that are targeted by the phage, which influence the phage transcriptional landscape. These mechanisms include prophage induction, bacterial receptors shutdown, and motility inhibition. In addition, specific transcriptional events were captured for specific growth conditions, indicating that phage can adapt the bacterial cell to reallocate resources for infection and surpass the limitations imposed by bacterial defences. The results put into evidence the relevance of using complex settings that mimics *in vivo* conditions to study phage-bacteria interplay, and the obviously phage versatility on bacterial cell invasion under different environments. Besides providing a better understanding on phage/host interactions, transcriptomics did not disclose the phage's genes that are essential for phage replication. Therefore, a genomic approach based on the direct manipulation of phage's genome was made to obtain phages with a reduced genome. This approach consists in exposing the phage to a mutagenic agent and heat treatments in the presence of a chelating agent. The surviving phages had large deletions (up to 3kb) in their early genes and revealed similar ability in the killing of the bacterial host. However, major changes occur in terms of the host range and in their long-term competitiveness, which might indicate that early genes are not essential for phage replication in lab conditions but confer competitive advantages to the phage. This approach enables the fast generation of mutants that can provide clues towards a better understanding of the role of unknown genes and potentially drive the discovery of novel phage sequences and phage core infection mechanisms at the molecular level. **Keywords:** phage; RNA-seq; *P. aeruginosa*.

RESUMO

Os fagos são vírus que infetam bactérias e que têm elevado interesse devido à sua aplicabilidade nas áreas da saúde humana, biotecnologia, agricultura, veterinária, e biologia sintética. Como agentes terapêuticos, os fagos podem ser usados para tratar infecções provocadas por bactérias resilientes, como *P. aeruginosa*. Assim, a sua aprovação está sobretudo limitada à falta de conhecimento no modo de ação dos fagos em condições *in vivo* e nas respostas bacterianas associadas. Neste trabalho foi realizado RNA-seq de culturas bacterianas infetadas com fago que cresceram em dois meios distintos, e aderidas a epitélio de pulmão humano, para obter uma visão global dos eventos transcricionais que ocorrem durante a terapia fágica. Assim, foi possível provar que *P. aeruginosa* encontra-se significativamente mais virulenta quando cultivada em meio de crescimento de células epiteliais, assim como quando aderida a uma monocamada das mesmas, comparativamente em meio “Lysogeny broth”. Durante a infecção do fago, o transcriptoma global é muito diferente na fase inicial da infecção, etapa importante em que o fago faz uma reprogramação da célula bacteriana com alterações em metabolismos associados à produção de energia. Curiosamente, em todas as condições, há um conjunto de mecanismos comuns que são manipulados pelo fago e influenciam o transcriptoma bacteriano. Estes incluem a indução de profago, diminuição da expressão de recetores da superfície bacteriana, e inibição da motilidade. Ainda, eventos transcricionais específicos foram capturados em determinadas condições de crescimento, indicando que o fago adapta a célula bacteriana para realocar recursos essenciais à infecção e ultrapassar as limitações impostas pelas defesas bacterianas. Os resultados põem em evidência a relevância de usar condições complexas que mimetizem as condições *in vivo* para estudar a interação fago-bactéria, assim como a óbvia versatilidade do fago em invadir a célula bacteriana em diferentes condições. Apesar de permitir conhecer a interação fagos/hospedeiro, os dados obtidos não permitiram inferir sobre os genes dos fagos essenciais para a sua replicação. Assim, recorreu-se a uma abordagem genómica para gerar fagos com genomas reduzidos. Esta abordagem consistiu em expor os fagos a um agente mutagénico e um tratamento térmico na presença de agente quelante. Os fagos sobreviventes possuem deleções consideráveis e são eficientes contra a bactéria hospedeira. Contudo, mudanças significativas ocorrem no espectro lítico do fago e na sua capacidade competitiva a longo termo, indicando que os genes iniciais não são essenciais para a replicação do fago em condições laboratoriais, mas conferem vantagens competitivas ao fago. O método permitiu gerar mutantes que providenciem conhecimento sobre o papel de genes de fago desconhecidos, e potencialmente descobrir novas sequências de fago e elucidar mecanismos de infecção do fago a nível molecular. **Palavras-chave:** fago; RNA-seq; *P. aeruginosa*.

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LIST OF ABBREVIATIONS

AMGs —Auxiliary metabolic genes
ANOVA — Analysis of variance
Apr — Alkaline protease secretion system
Aqs1 — Quorum-sensing anti-activator protein
ATPase — Adenosine triphosphatase
ATUn — Temporary Authorization for Use
bp — Base pair
BRED — Bacteriophage recombineering of electroporated DNA
Cas — CRISPR Associated (proteins)
CDC — Centers for Disease Control and Prevention
c-di-GMP — Bis-(3'-5')-cyclic dimeric guanosine monophosphate
cDNA — Complementary DNA
CDS — Coding DNA Sequence
CF — Cystic Fibrosis
CFU — Colony forming unit
COGs — Clusters of Orthologous Groups
CRISPR — Clustered Regularly Interspersed Palindromic Repeats
DEG — Differential Expression Genes
DNA — Deoxyribonucleic acid
dRNA-seq — Differential RNA-seq
DRS — Helicos Direct RNA Sequencing
ds — Double-stranded
eDNA — Extracellular DNA
EDTA — Ethylenediamine tetracetic acid
EEA — European Economic Area
eIND — Emergency investigational new drug pathway
EOP — Efficiency of plating
EPS — Extracellular polymeric substances
FDA — Food and Drug Administration
FISH — Fluorescence in situ hybridization

FRT-seq — Illumina-based RNA-seq approach in which reverse transcription takes place on the flow cell surface

GC-content — Guanine-cytosine content

GCP — Good clinical practices

gDNA — Genomic DNA

GMP — Good manufacturing practices

gp — Gene product

HA — Hydroxylamine

HAI — Healthcare-associated infection

HGT — Horizontal genetic transfer

HIV — Human immunodeficiency virus

ICTV — International Committee on Taxonomy of Viruses

ICU — Intensive-care units

IDR — Innate defence regulatory peptides

kb — kilobase

LB — Lysogeny Broth

LPS — Lipopolysaccharide

MCCM — Mammalian Cell Culture Medium

MDR — Multidrug resistant

Mip —MvaT inhibiting protein

MOI — Multiplicity of infection

mRNA — messenger RNA

MRSA — Methicillin resistant *Staphylococcus aureus*

ncRNA — Non-coding RNA

nt — Nucleotide

OD — Optical density

OMPs — Outer membrane proteins

OMVs — Outer membrane vesicles.

ONT-cappable-seq — Nanopore-based cDNA Cappable-seq approach

ORF — Open Reading Frame

PBS — Phosphate-buffered saline

PCA — Principal Component Analysis

PCR — Polymerase chain reaction
PEG — Polyethylene glycol
PFU — Plaque forming units
poly(dT) — Poly (deoxyadenylate Deoxythymidylate
PQS — Pseudomonas quinolone signal
qPCR — Quantitative polymerase chain reaction
QS — Quorum sensing
Qst — Quorum sensing targeting protein
RBPs — Receptor binding proteins
RBS — Ribosome binding site
RIN — RNA integrity number
R-M — Restriction-modification systems
RNA — Ribonucleic acid
RNAP — RNA polymerase
RNA-seq — RNA sequencing
ROS — Reactive oxygen species
rpm — Rotations per minute
rRNA — Ribosomal RNA
RT — reverse transcriptase
RT-qPCR — Real-Time Quantitative Reverse Transcription PCR
SNAMPs — synthetic mimics of antimicrobial peptides
SNP —Single nucleotide polymorphisms
ss — Single-stranded
T1SS — Type I secretion system
T2SS — Type II secretion system
T3SS — Type III secretion system
T4SS — Type IV secretion system
T5SS — Type V secretion system
T6SS — Type VI secretion system
TCA — Tricarboxylic acid cycle
tRNA — Transfer RNA
TSS — Transcription start sites

US — United States

UTIs — Urinary tract infections

VBNC — Viable but non-culturable cells

vRNAP — Virion RNAP

WHO — World Health Organization

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SCIENTIFIC OUTPUTS

Papers in peer reviewed journals:

- **Brandão, A.**, Putzeys, L., Pires, D.P., Voet, M., Paeshuyse, J., Lavigne, R., and Azeredo, J. (2022) Impact of phage predation on *P. aeruginosa* adhered to human airway epithelium: major transcriptomic changes in metabolism and virulence-associated genes. Under review in RNA Biology.
- **Brandão, A.**, Pires, D.P., Coppens, L., Voet, M., Lavigne, R., and Azeredo, J. (2021) Differential transcription profiling of the phage LUZ19 infection process in different growth media. RNA Biol 00: 1–13.
- Pires, D.P., Meneses, L., **Brandão, A.**, and Azeredo, J. (2022) An overview of the current state of phage therapy for the treatment of biofilm-related infections. Curr Opin Virol 53: 1–7.

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- Brandão, Ana; Pires, Diana; Voet, Marleen; Coppens, Lucas; Lavigne, Rob; Azeredo, Joana. “Differential Gene Expression Analysis of LUZ19 Phage and *Pseudomonas* Growing in Epithelial Cell Culture Medium vs Luria Broth Medium”. Oral presentation in Section IV: Phage-Host Interactions at 23rd Biennial Evergreen International Phage Meeting, 2019.
- Brandão, Ana; Pires, Diana; Voet, Marleen; Coppens, Lucas; Lavigne, Rob; Azeredo, Joana. “Differential transcription profiling of the phage LUZ19 infection process in different growth media”. Oral presentation in Health Biotechnology @ CEB session of Center of Biological Engineering Biennial Scientific Meeting, Braga, 16th May, 2022.

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CHAPTER I: RESEARCH GOALS AND THESIS OUTLINE

Bacteriophages (hereinafter designated by phages) are intracellular parasites that usurp cell machinery for their own propagation. The mechanisms behind bacterial cell reprogramming during phage infection are poorly known, as well as the function of many early genes annotated as hypothetical proteins. Transcriptomics is a powerful tool to study phage-host interactions and has been widely applied to discover regulatory sequences and the role of phage genes. However, the phage biology knowledge has been limited to studies of host-phage interactions under well-controlled laboratory conditions that fail to mimic the complexity of microbial communities in real environments. From a phage therapy perspective, it is important to understand how phages interact with their bacterial hosts in human physiological conditions. Thus, the main goal of the work described in this thesis was to elucidate the interaction between a potential therapeutic phage, the *Pseudomonas* LUZ19 phage, and its host *P. aeruginosa* PAO1, a pathogen responsible for many difficult-to-treat infections, such as lung infections.

This thesis is divided in 5 chapters, each addressing specific aims of the work. **Chapter II:** aims at providing an overview of the current knowledge about phage-host interaction in general and specifically the interplay between *P. aeruginosa* and its infecting phages in a context of phage therapy. **Chapter III:** aims at disclosing the influence of bacterial growth media in capturing phage-bacteria interactions at the transcriptional level, and how the media affects both phage and bacteria transcriptomes. It also aims at understanding phage-host interaction in more complex conditions, such as in the presence of a medium that supports the growth of human airway epithelial cells (Mammalian Cell Culture Medium, MCCM medium). To assess that, RNA-seq of synchronized phage LUZ19 infected cultures of *P. aeruginosa* PAO1 in LB medium and MCCM medium was performed. **Chapter IV:** aims at understanding phage-bacteria interactions under conditions that mimics *P. aeruginosa*-associated infections in the human lung. To achieve that, transcriptome analysis of a complex mixture containing human lung epithelial cells-bacteria-phage was performed. **Chapter V** aims at identifying in phage's genome, genes that are not essential for phage particle viability. To achieve that, a simple, non-cost, and fast method was used, which was based on the combination of a chelating agent with heat treatment to select thermostable phages with large deletions in the genome. Together with transcriptomic data, the results described in chapter V contributed to a better elucidation of phage-bacteria interactions, and give clues towards phage genomes manipulation to achieve safer and efficient phages, for instance for phage therapy purposes.

CHAPTER II: LITERATURE REVIEW

This chapter contain information on phage efficacy against *in vitro*-formed biofilms that was retrieved from following article:

— Pires, D.P., Meneses, L., **Brandão, A.**, and Azeredo, J. (2022) An overview of the current state of phage therapy for the treatment of biofilm-related infections. *Curr Opin Virol* 53: 1–7.

Brandão, A. contributed for the writing of this article and kindly acknowledge to remaining authors of the above-mentioned review article for the opportunity to contribute to its writing.

2.1 *Pseudomonas aeruginosa*

2.1.1 A worldwide public health concern

Antimicrobial resistance (AMR) poses a serious global threat of growing concern. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. Bacteria from the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) are major nosocomial pathogens that quickly acquire resistance to multiple drugs and therefore present a major health concern [1].

P. aeruginosa bacteria has been in the spotlight since 2017, when it was identified by the World Health Organization (WHO) as one of the top three pathogens urgently requiring the development of alternative treatments. Its classification as a critical pathogen is attributed to the high number of reported infection cases caused by multidrug-resistant (MDR) isolates [2]. MDR *P. aeruginosa* was also recognized by the Centers for Disease Control and Prevention (CDC) as a serious threat in the United States (US), causing about 32,600 healthcare-associated infections, 2,400 deaths, and loss of 767 million dollars in the health care system in 2017 [3]. The annual epidemiological report of 2020 on antimicrobial resistance in the EU/EEA revealed that *P. aeruginosa* was one of the commonly reported species (6.2%), and carbapenem resistance was shown to be a very common trait among clinical isolates [4]. A greater concern is with the patients that stay for more than two days in intensive-care units (ICU). In 2017 at least 8.7% of these patients acquired healthcare-associated infections (HAI) that require (prolonged) ICU stays [5]. According to ECDC Annual Epidemiological Report [5], the most prevalent HAI in Europe in 2017 was pneumonia (6%), in which *P. aeruginosa* was the most prevalent isolated microorganism (19.9%) with a 26% incidence of carbapenem-resistant isolates. The same report demonstrated that from the ICU-acquired bloodstream infections, *P. aeruginosa* was responsible for 9.5% of cases and was also the fourth most prevalent organism isolated from ICU-acquired urinary tract infections (UTIs) (14.5%) [5]. From a general trend, 26.5% of *P. aeruginosa* isolates were resistant to ceftazidime and 25.9% resistant to carbapenem antibiotic [5].

Considering the worrisome increase of *P. aeruginosa*-associated infections, this pathogen has been intensively studied to understand its ubiquity in nature, resilience, and ability to acquire resistance to multiple drugs.

2.1.2 General traits and infection mechanisms

P. aeruginosa is a Gram-negative bacterial pathogen that can survive and colonize several habitats, including soil, marine habitats, non-living surfaces, plants, animals, and human tissues [6–8], revealing an incredible adaptability to diverse environmental conditions. It is a rod-shaped, non-spore-forming, aerobic, monoflagellated bacterium that grows optimally between 25-37°C, although it can grow under anaerobic conditions and at 42°C [9,10]. This bacterium is an opportunistic pathogen responsible for serious nosocomial infections in immunocompromised hosts, including those with HIV, neutropenic cancer, metabolic disorders, and bone marrow donors. The most common infections are bacteraemia and sepsis in patients with burns, pneumonia, keratitis, urinary-tract infections in catheterized patients, infections following surgery, and chronic pulmonary inflammation in cystic fibrosis (CF) patients. *P. aeruginosa* can also lead to mild illness in healthy individuals, including otitis and contact lenses-derived infections [6,8,11–15]. In adult patients with CF, *P. aeruginosa* is the most prevalent isolated pathogen from the respiratory tract and it is also the major cause of morbidity and mortality in these patients [16,17]. In addition, *P. aeruginosa* is also the major pathogen isolated from non-CF bronchiectasis [18].

Considering the major prevalence of *P. aeruginosa* in lung infections, it is important to understand its mechanisms of infection. The first step of the respiratory infection begins with the bacterial attachment to lung epithelial cell receptors mediated by bacterial adhesins, the flagella, and pili. Also, these bacteria secrete toxins to induce cell damage and produce an extracellular matrix, enabling bacteria to form a biofilm that facilitates bacterial adhesion to lung epithelium, protection from the immune system and antibiotics. In chronic infections, *P. aeruginosa* forms thick biofilms in the airway mucus and often mutates to acquire a mucoid phenotype without adhesins and with high production of alginate (an exopolysaccharide). This provides additional protection to the biofilm from the immune system and antimicrobials, causing this mucoid phenotype to become very common in the isolates from CF patients [18]. Overall, several intrinsic mechanisms contribute to the infection success of *P. aeruginosa* bacteria, which is equipped with a group of virulence factors and pathogenicity mechanisms.

2.1.3 Virulence, cell communication and biofilm formation

One of the bacterial factors that provide the highly versatility and adaptability is the large size and complexity of the *P. aeruginosa* genome, which is thought to provide bacterial flexibility to survive in diverse environments [6]. Gene expression control in this bacterium is a key determinant of its adaptability and flexibility to numerous environments, with 8.4% of its genes involved in genome regulation. From its genome, 54.2% of genes encode known open reading frames (ORFs), and most of them are associated

with lipopolysaccharide (LPS), biosynthetic enzymes, virulence factors, including exoenzymes and their secretion systems, and genes involved in motility and adhesion [6,15]. Apart from *P. aeruginosa* core genome sequence, each isolate contains a very diverse “accessory genome” that encode a variety of genetic elements associated with transposons, prophages, insertion/integration/replacement of genetic sequences, which might be involved in the adaptation of this species to several niches [19]. Apart from the diversity of the *P. aeruginosa* “pangenome”, each strain is considerably complex, possessing numerous regulatory mechanisms that allow bacterial adaptation under environmental changes, such as observed by Dötsch and collaborators who showed that under varying bacterial growth conditions, a single strain constantly transcribes only 46% of its genes [20].

The virulence of *P. aeruginosa* is multifaceted, involving virulence factors and secondary metabolites production, cell-associated factors, nutrient scavenging, motility, and biofilm formation [11,14,21]. The most important virulence factors include cytotoxins (ExoU, ExoT, ExoS, and ExoY) produced by type III secretion system (T3SS) that are injected into the host cell in order to avoid bacterial clearance, and pyocyanin that causes tissue damage and impairs organ’s proper function. Tissue damage can also be mediated by rhamnolipids by the degradation of lung surfactant and tight junctions from host cells. This orchestration of the virulence factors can be achieved by a group of enzymes that comprises LasA and LasB elastases, alkaline protease (AprA), LipC lipases, phospholipase C, and esterase A. In addition, *P. aeruginosa* has other ways to exert its virulence, for instance by expressing siderophores (pyoverdine and pyochelin) to increase the ability to uptake iron in environments with a low concentration of nutrients, or by expressing antioxidant enzymes (catalases-KatA, KatB, and KatE, alkyl hydroperoxide reductases, and superoxide dismutases) to escape from reactive oxygen species (ROS) produced by host innate immune cells [22].

In terms of cell-associated factors, the LPS are one of the most important surface components of *P. aeruginosa*, known to have a role in host cells receptor recognition, attachment, and endotoxicity-mediated tissue damage. Moreover, it is also associated with antibiotic tolerance, and biofilm formation. Other cell-associated factors are the outer membrane proteins (OMPs) that are involved in bacterial adhesion, nutrient exchange, and also antibiotic resistance. In addition, also the flagellum, pili, fimbriae, and other adhesins are involved in cell adhesion. *P. aeruginosa* can also produce outer membrane vesicles (OMVs) to deliver a variety of virulence factors into lung epithelial cells without direct contact [22].

The virulence factors are secreted by different secretion systems, grouped as six types in *P. aeruginosa*: the TSS1 is involved in the Apr secretion system and Has secretion system (involves secretion

of an extracellular haem-binding protein (hasA) that regulates iron metabolism); T2SS (secretion of guanylate cyclase ExoA, proteases LasA/B, and multiple other factors); T3SS involved in all machinery that produces and delivers exotoxins; T4SS (involved in horizontal genetic transfer (HGT)); T5SS (secretion of proteins such as EstA, LepB, and LepA); and T6SS (secretion of TseF to facilitate the import of the *Pseudomonas* quinolone signal (PQS)-Fe³⁺, deliver of antibacterial toxin Tle3, among others) [10,22].

Most of these virulence mechanisms are time-coordinated by activating or repressing target genes in response to the external signals by a mechanism of cell communication called quorum sensing (QS), where a cell senses the aspects of the external environment and adapts the expression of its genes as well as expresses products (autoinducers) that will function as signalling molecules to adapt cell population gene expression, and therefore pathogenicity. As such, QS has been reported to be involved in the i) bacterial ability to resist antibiotics, ii) ability to initiate the infection and persist in the host, and iii) biofilm formation [7,11–15,21].

The ability to form biofilms is one of *P. aeruginosa* characteristics implicated in chronic infections. In the biofilm, the bacterial community is irregularly structured as surfaced adherent microbial communities, in which cells are embedded in a matrix of extracellular polymeric substances (EPS) that protects them from antimicrobial treatment and the host immune system [23–26]. The EPS matrix is usually composed of polysaccharides, proteins, extracellular DNA (eDNA), and lipids. Alginate is the most important polysaccharide found within the matrix of *P. aeruginosa* biofilms and is commonly produced by mucoid *P. aeruginosa* strains found in CF patients [18]. Besides protection guaranteed by the structural nature of the biofilm, in this lifestyle, the microbial population is in different growth states and display different phenotypes. For instance, *P. aeruginosa* biofilms are highly tolerant to antimicrobials due to difficulty of antimicrobials to penetrate in these structures, but also because the inner layers of biofilms contain metabolically inactive cells that consequently better tolerate antibiotics. Thus, antimicrobials target only a biofilm sub-population that is metabolically active (failing to kill dormant bacteria). In addition, bacteria trigger differential expression of multiple gene networks that allows the biofilm population to better tolerate antimicrobials [27]. This makes biofilm itself a more resilient microbial “living mode” compared to logarithmic-phase planktonic cultures [24,25,28,29]. Nevertheless, the bacteria have the capacity to change between free-living (planktonic) and biofilm (surface-attached) lifestyles, allowing an increased capacity to adapt and survive in several environments [15] (Figure 2.1).

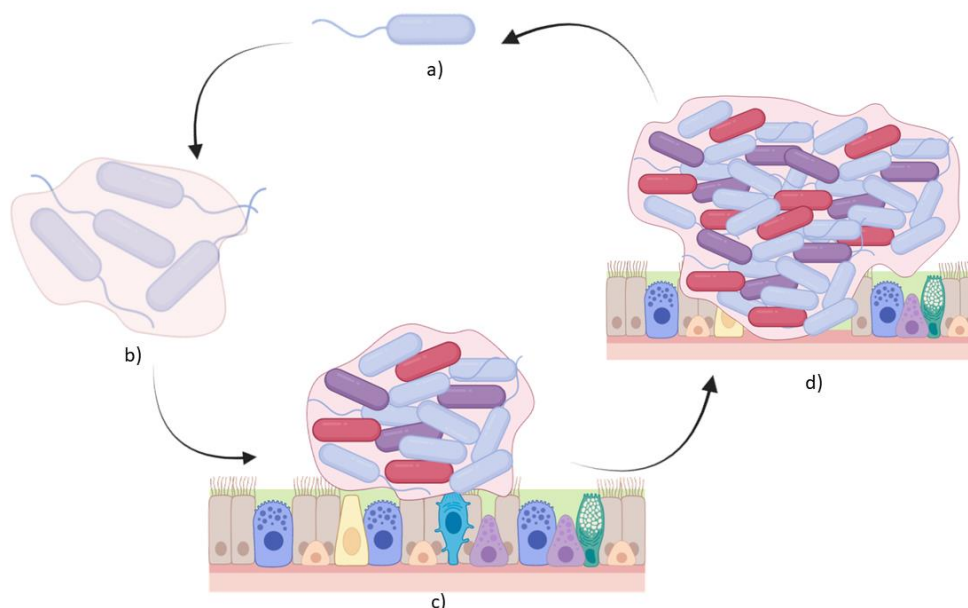


Figure 2.1- Main steps of bacterial biofilm formation: a) Free-swimming cells in a planktonic state begin to aggregate to each other and form cell aggregates in suspension (b), which usually tend to adhere to surfaces such as epithelial lung layers mediated by cell adhesins (c), that attach and recognize host surface receptors. In acute stages, bacteria release virulence factors through different secretion systems to promote successful infection and multiplication until the formation of a mature biofilm, composed of a thick mixture of bacteria in different metabolic states and phenotypes embedded in a complex biofilm matrix. This last stage is the predominant lifestyle of the bacterial community found during chronic infection stages. Here, it is possible that some bacteria detach and colonize other sites, leading to the spread of the infection.

2.1.4 Multidrug resistance mechanisms

Treatment of nosocomial infections caused by *P. aeruginosa* is strongly threatened by the constant emergence of antibiotic-resistant variants and by the difficulty of finding new antibiotics [30–32]. This bacterium has already developed resistance to several β -lactams including carbapenems (imipenem, meropenem), cephalosporins (ceftazidime), and monobactams (aztreonam). In addition, other antibiotic classes like aminoglycosides (gentamycin, tobramycin, amikacin), fluoroquinolones (ciprofloxacin), and polymyxins (colistin, polymyxin B) have been included in the *P. aeruginosa* antimicrobials resistance range [33].

P. aeruginosa resistance is complex and relies on the synergy between intrinsic, adaptive, and acquired resistance mechanisms. Intrinsic resistance is related to the reduced ability that wildtype bacteria have to uptake antibiotics through the outer membrane, especially for small hydrophilic

antibiotics (such as β -lactams and quinolone that pass slowly through porin channels). Additionally, bacteria have multidrug efflux pumps that actively pump antibiotics as they pass through, and have periplasmatic enzymes (like β -lactamases) that degrade or inactivate antibiotics [34]. In turn, acquired resistance is a result of spontaneous genetic mutations, or it can arise through the acquisition of resistance determinants by horizontal transfer of mobile genetic elements (e.g., plasmids, transposons, interposons, and integrons) via transduction, transformation, and/or conjugation [27,34,35]. Additionally, bacterial resistance can be a transient mechanism that is induced by variation of growth conditions (e.g., nutrients available, biofilm formation and swarming motility, environmental stimulation, continuous and inappropriate exposure to sub-inhibitory antibiotic concentrations, stress caused by pH and heat shock variations, DNA stress, and anaerobiosis) being usually reported as an adaptive resistance [36,37]. The small fraction of cells surviving antibiotic treatment due to non-mutational mechanisms are called “persister cells”, and these constitute a phenotypic variation where the cells are metabolically inactive and present less transcription, translation, and replication of the genome. These characteristics allow them to be highly tolerant to antibiotic treatment but once the antibiotic therapy is stopped, “persister cells” become metabolically active again [38]. “Persister cells” are highly prevalent in *P. aeruginosa* biofilms and are responsible for the high tolerance of biofilms to antibiotics together with the diffusional limitations imposed by the matrix and high population heterogeneity, as discussed above. Thus, eradication of *P. aeruginosa* biofilms is difficult to achieve with antibiotics since the bacterium that escape from these treatments become fully prepared to resist subsequent treatments and the small fraction of survived-population quickly leads to the beginning of a well-established disease and therefore, antibiotic treatment leads to an active selection for MDR *P. aeruginosa* isolates. Thus, there is an urgent need to develop new strategies to combat *P. aeruginosa* infectious diseases, particularly those associated with biofilm formation [39,40].

2.1.5 Strategies to control *P. aeruginosa* infections

A common therapeutic approach consists of combined antibiotic therapy because it extends the effective life of each used antibiotic, it has a synergistic effect and reduces the development of antibiotic resistance. However, synergy is rarely tested in the biofilm state and its understanding requires deeper knowledge, particularly extensive work is necessary to optimize the combination and concentration of each antibiotic. Besides the apparent success of antibiotics conjugation therapy, biofilms have a resilient nature and multi-drug resistance, being still difficult to eradicate [41].

Several approaches have been developed to minimize the infections caused by *P. aeruginosa*, including the use of gene editing (e.g., CRISPR/Cas9), antimicrobial peptides, bacteriocins, probiotics, vaccines, synthetic mimics of antimicrobial peptides (SNAMPs), innate defence regulatory (IDR) peptides, anti-virulence products (e.g., QS inhibitors), and nanoparticles [42,43]. In addition, there is a naturally occurring alternative, the use of phages or phage-derived products. Phage research gained a renewed interest once these have been pointed out as one of the best solutions to fight MDR bacteria and work as antibiotic substitutes. The next section describes in detail phage basic biology as a promising alternative to fight *P. aeruginosa*-associated infections.

2.2 Phages: Viruses of bacteria

Phages are small viruses that specifically prey prokaryotes for their replication, being the most abundant entities in nature that can be found in several habitats [44,45]. Phages are known to be harmless to humans, plants, and animals, and are even considered as important ecosystem shapers in the sense that, phages modulate bacterial abundance and evolution in microbial communities at a global and local scale [46]. Their discovery was made independently by Frederic Twort (1915) and Félix d'Hérelle (1917) [44], where D'Hérelle was the first one to suggest that phages are bacterial viruses. Since that time, phage research has evolved over more than one century, laying the foundation for molecular biology and becoming one of the first approaches to combat human infections caused by bacterial pathogens.

2.2.1 Life cycle and classification

Virulent phages with a strictly lytic cycle begin their infection by adsorbing to bacterial cell surface receptors, performing an attachment that allows them to inject their genetic material into the host cytoplasm. Here, host replication, transcription, and translation machinery are redirected to produce new phage particles that are released at the end of the infection cycle through the action of phage enzymes that led to cell lysis. Temperate phages, on the other hand, can integrate their genomic material into the bacterial genome, remaining as a prophage (without production of progeny and consequently bacterial lysis) and replicate together with the bacterial genome for several generations, until the lytic cycle is induced by an external, stress-related stimulus [46,47]. In addition, phages can also adopt other life cycles described as pseudolysogeny (phage doesn't integrate into the bacterial chromosome and is transmitted to daughter cells), carrier state (large production of phages and accumulation into the cell without release), and chronic infection (phage release without cell lysis) [47,48] (Figure 2.2).

Phage diversities are also extended to its genomic traits and morphology, based on a classification established by the International Committee on Taxonomy of Viruses (ICTV), which comprises their morphology (size and shape), genetic material, life cycle, host, proteomic and genomic analysis [49]. The most abundant phages in nature are tailed phages with double-stranded DNA (dsDNA) genome, which belong to *Caudoviricetes* class [50]. Tailed phages have a similar morphology in terms of possessing an icosahedral capsid containing genomic material (together they constitute the “head”), a head-to-tail connector, and a tail, being the last trait that differentiates these phage families. In terms of morphology, phages are usually divided into three morphotypes: a long-rigid-contractile tail with a sheath around the tail tube (myovirus), a long-flexible-non-contractile tail (siphovirus), and a short tail (podovirus) [51].

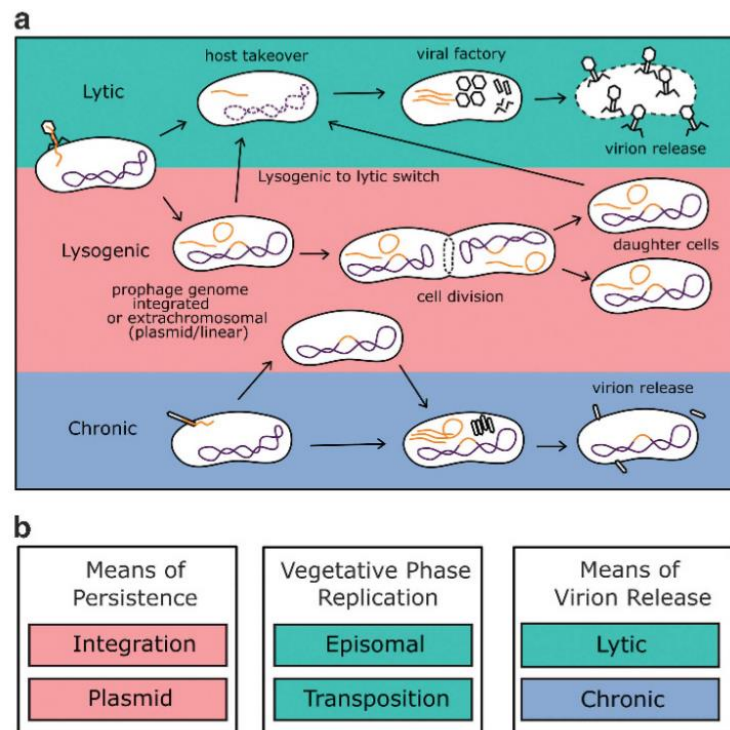


Figure 2.2- Phage life cycles (lytic, lysogenic, pseudolysogenic, chronic infection). a) In lytic cycle phages infect bacteria and produce new virions that are released in the end-of-life cycle as a result of cell lysis; on the other side, in lysogenic life cycle phage genome can be integrated in bacterial chromosome and passed to bacterial daughter cells until a signal stimulates phage release and lytic life cycle progress. Related to lysogenic cycle, pseudo-lysogenic life cycle is referred to phages that are maintained and transmitted to next bacterial generations as plasmids or episomes. Differently from other life cycles, in the chronic life cycle the phage virions are released without bacteria lysis, which is the opposite of carrier state (not represented), where virions are produced but these are accumulated into the bacterial cell. b) different life cycles can be distinguished by means of persistence, replication and

virions release, being highlighted these traits for the different life cycles. The image was adapted from [48].

2.2.2 A brief history of phage therapy

Natural phage therapy consists of using strictly lytic phages to fight bacterial infections. Phage's first application as a therapeutic agent began in 1919 to treat dysentery, and after that, they were mostly applied in Eastern Europe (Poland and the Soviet Union). In the West, phage therapy was largely abandoned due to the rise of the “antibiotics-era” and due to the lack of knowledge about phages basic biology [44,52]. With the emergence of MDR isolates and the increase of outbreaks caused by resistant bacteria, a renewed interest for phage therapy has emerged in the last two decades as a promising adjunct to antibiotics. A basic rationale to revisit phage therapy was because of the multiple advantages of phages over antibiotics, such as rapid, easy, and inexpensive phage production; high level of specificity, which implies a reduced impact in human microbiota; harmlessness to eukaryotic cells [52]; and minimal side effects and self-replication [44,53]. As a consequence, the number of studies demonstrating phages' safety and efficacy against human diseases has increased. A special relevance is made to the studies that take into account the infections driven by biofilms.

2.2.3 Phage efficacy against biofilms: Lessons from *in vitro* assays

Most of the studies begin by using *in vitro* evaluation of phage efficacy against biofilm-related infections, which usually consists of studying mono-species biofilms formed on polystyrene microtiter plates and testing single phage preparations, phage cocktails, or phages combined with antibiotics to assess its efficacy by cell viability, biomass quantification, metabolic activity, or microscopic visualization [54]. In some cases, an attempt to simulate real infections is made by using other types of materials inside the microtiter plates including, stainless steel coupons [55], catheter sections [56,57], collagen [9] or human cells monolayers [59]. However, the *in vivo* complexity of biofilms in human infections is not well mimicked in these assays, since clinical biofilms harbour a complex and heterogeneous community with multiple bacterial species and sometimes even fungi [60]. Even by performing mixed biofilms (typically dual-species biofilms), *in vitro* models are not entirely capable of reproducing real infection conditions. Therefore, the translation of *in vitro* results into clinical outcomes is not straightforward. Indeed, in addition to harbouring a heterogenous polymicrobial community (including viable but non-culturable cells-VBNC), other factors might influence phage action on real biofilms. For instance, *in vivo* stressors/inhibitory compounds, bacteria under different metabolic states, fluids

dynamics, and host immune system are factors that are not reproduced by *in vitro* methods. Nonetheless, all possible knowledge acquired from the *in vitro* studies can be a major advantage to investigate the variables that rule biofilm control by phages and understanding the potential of phage therapy. Thus, *in vitro* biofilm-forming methods are useful to assess the biofilm-killing efficacy of phages alone or combined with other antimicrobial agents; to test phage formulations *in vitro* (to understand its release, dose, and stability) before doing *in vivo* tests; administration strategy (simultaneously vs. sequential) [61,62]; to predict the emergence of phage-resistant variants (related with bacterial modifications on phage receptors [11,12], defective growth, reduced virulence [15], re-sensitization to antibiotics [65,66] and the immune system [67]); its long-term implication in the therapeutic context (for instance, increase biofilm thickness due to the release of extracellular DNA under lower levels of phage predation [68]); and to study phage-antibiotics synergy [62,69–73]. However, extrapolations towards the outcomes for phage-antibiotic combinations should be carefully addressed since many factors may influence the phage-antibiotic synergy such as the antibiotic class, the *in vivo* biological environment (for example, the presence of human serum), phage species, as well as phage and antibiotic concentrations [24]. It is also important to consider the long-term effects of the phage-antibiotics combination, since there are reports suggesting that the accumulation of extracellular DNA ultimately leads to thicker biofilms and that antibiotics can function as communication molecules to modulate microbial communities [75]. This draws attention to the need for improved *in vitro* methods that better assess phage formulations and mimic long-term infections by using dynamic conditions or surfaces that simulate human tissue/device surfaces.

Considering the limitations of using *in vitro* methods to guarantee phage efficacy and safety, it has been observed an increased number of *in vivo* studies performed in animal models, as well as an increasing number of clinical trials and clinical cases in humans.

2.2.4 *In vivo* studies, clinical cases and clinical trials using phages to fight *P. aeruginosa*

Positive results obtained from laboratory studies allowed the expansion of phage therapy in western Europe. Recently, case studies focused on the use of phages to treat *P. aeruginosa* infections in humans have been published and, in general, most of them report infection resolution or a clinical improvement [76–90]. An increasing number of studies have also demonstrated the phage's ability to kill *P. aeruginosa* *in vivo* using different animal models, as reviewed by Melo and collaborators [91]. To date, the application of phages against *P. aeruginosa* infections were previously studied in the following animal models: mouse model of respiratory infection [92,93], mice models of bacteraemia, lung and wound

infections [94–98], keratitis model in rabbits [99], sheep sinusitis model [100], porcine model [101] and *Galleria mellonella* infection models [102]. These studies highlight that phage researchers are now addressing the importance to test and develop phage products *in vivo* before jumping to precipitated conclusions given by *in vitro* methods and testing on humans. This has prompted the emergence of clinical trials that would be useful for validating phages as therapeutic agents.

The first clinical trial used to treat *P. aeruginosa* infections with phages was done by *Wright et al.* in 2009 and consisted in applying a six-phage cocktail to treat chronic *P. aeruginosa* otitis. In this trial, the results demonstrated a significant reduction of bacterial counts in the phage-treated group, while in the placebo group there was no significant bacterial reduction. In general, there were no significant side effects in both groups, which sounded a promise of phage therapy success and safety [103]. However, recently it was published the results from a randomized phase I/II trial where a cocktail of lytic *P. aeruginosa* phages was used to treat wound infections, which was the first trial made under good manufacturing practices (GMP) and good clinical practices (GCP). This trial was stopped due to insufficient efficacy of the cocktail since during the follow-up period, isolated bacteria revealed to be resistant to phage. Another limitation was associated with adverse effects that were observed (23%) and with the shelf-life of the phage cocktail that was very reduced [104]. At the moment, there are several phase I/II trials going on and planned to start this year, which will evaluate different phage products against *P. aeruginosa*: the APPA02 cocktail to treat serious respiratory infections (NCT04596319), YPT01 cocktail to treat chronic *P. aeruginosa* infections in CF (NCT04684641), phage cocktail TP102 to treat multispecies infections on diabetic foot ulcers (NCT04803708), BX004-A (NCT05010577), PL03BM (NCT04815798) and PGX0100 (NCT04323475) [105].

This evolution in the development of phage-based products has been driven by the development of dedicated protocols for phage therapy in humans. For instance, in Belgium, a national regulation for magistral preparation was implemented for tailor-made phage medicine to treat individual patients [106]. Similarly, in France, phages can be used under the nominative Temporary Authorization for Use (ATUn), and in the United States through FDA emergency investigational new drug (eIND) pathway [107].

2.2.5 Constraints of phage therapy

Although, considering all the advantages of using phages that were already mentioned above, there are also some limitations associated to phage therapy: less accessibility of intracellular bacterial pathogens to phages; narrow host range; possible phage clearance by the immune system, especially when applied using higher phage titers; release of intracellular toxic components like bacterial endotoxins

after cell lysis; emergence of phage-resistant bacteria during treatment; diffusional limitations of phages through the biofilm matrix and low metabolic activity of biofilm cells; inhibition of phage infection by QS; and the need for phage production according to GMP guidelines and in large amounts [44,45] (with exception of magistral preparations in Belgium). In addition, there are still regulatory constraints to phage application, because most of the genes encoded in phage genomes are still of unknown function [52,108], and many phages can carry in their genomes genes that encode for virulent or toxic products, antibiotic resistance, and lysogeny.

Besides these disadvantages there is the potential chance to isolate new phages with novel features, such as encoding proteins that grants phage higher efficiency against biofilms, or for instance that infect a high panel of bacterial isolates, but also novel phages that might have compact genomes free of genes that codify for toxins or genes associated to genetic material exchange, being thus safer for therapeutic purposes. Besides, many phage therapy strategies had emerged to better overcome the barriers posed by biofilms, such as the use of mechanical debridement; dispersing enzymes; antimicrobial phage-derived enzymes; the use of phage cocktails to increase the spectrum of activity; and exploiting phage synergy with other antimicrobials [45,52].

In an ultimate approach, phages can be genetically engineered towards acquiring new functionalities, including the expansion of their host range [109], delivering antimicrobials [110,111], changing phage life cycle from temperate to virulent phages [112], removal of virulence/toxic genes, to have smaller genomes without unknown ORFs [102], to encode CRISPR/Cas systems that target the bacteria [113,114] or even encoding genes that inhibit bacterial resistance to phages (e.g., anti-CRISPR proteins) [92], among others. Phage engineering opens a near future in which phages can be validated as a therapeutic agent and substitute the current antibiotic therapy. For that, biological properties of phages need to be fully elucidated, so that it can be understood the consequences of its applications and better predict the patients' outcomes.

2.3 Phage genome modification

The genetic modification of phages is an innovative strategy to improve several phage traits that limit its generalized application (reviewed in [115]). Hence, these genetically manipulated phages can be rebooted to fulfil the demands of numerous research fields, which has prompted the development itself of the available genome engineering and synthetic biology tools. To date, several phage-engineering techniques have emerged, including techniques based in Homologous Recombination [116–122],

Bacteriophage Recombineering of Electroporated DNA (BRED) [123–128], Rebuilding/Refactoring phage genomes *in vitro* [129], Whole Genome Synthesis [130–132], Yeast-based platform [118,133–135], Cell-free transcription-translation systems [136], CRISPR-Cas based techniques [137–144], and L-form bacteria [145]. Nevertheless, most of referred techniques still have several limitations, namely in the difficulty of screening recombinant phages [125], low recombination rates [120–122], low transformation efficiency [118,129,133–135], lack of available tools for phages that infect non-model bacteria, limitations in terms of genome size [129,130–132], location of protospacer adjacent motif (PAM) sequences that restrict the genetic modifications in the case of CRISPR-Cas systems [141,142,146], and in some cases modification of more than one sequence can only be performed in a sequential steps [147–150]. Phage genome modifications began with small edits of phage genomes such as point mutations using mutagenic agents. Later, after the discovery of specific phenomena such as recombination and transposon, other new methods have been developed. In the next subsections, the ancient techniques used to create phage mutants without using synthetic biology tools will be addressed.

2.3.1 Creation of phage libraries through mutational techniques

Currently, there is an urgent demand for techniques that can generate phage libraries in a high throughput manner. In addition to the above-mentioned techniques where a rational design is performed to achieve one or more modifications in phage genomes, introducing small changes in phage genomes is also possible through other standardized techniques.

The most often used approach is to perform phage genome mutagenesis randomly. This usually leads to point mutations that are spread across the entire genome and that, can or not, lead to new phage variants/impair phage particle viability. Because of this, they are often used to study phage evolutionary processes [151]. This can be easily performed through chemical mutation using mutagenic agents such as ethyl methanesulfonate (EMS) [151,152] and hydroxylamine (HA) [153,154], or it can be used, for instance, ultraviolet (UV) radiation [155,156]. The advantage of this technique is that it leads to semi-biased mutations, not depending on host replication. These mutations are influenced primarily by the mutagenic agent that might favour specific transitions or transversion mutations [157]. This approach is not suitable to insert or swap genes, nor for well-rationalized phage engineering aims. In addition to chemicals and UVs, mutations in the phage genome can also be achieved through several phage propagations in a host that harbours a vector that encodes proteins that damage DNA or reduce DNA replication fidelity, thus leading to phage genomic mutations after several passages [152]. Nevertheless, this last method limits phage engineering to a specific host, as well as generated phage libraries. In

general, the random mutagenesis techniques lead to high diversity in the generated libraries, which are not programmed, and if the frequency of the mutations is aimed to be studied, it will demand depth sequencing.

Phage-targeted mutagenesis is also possible and is usually restricted to a specific locus. This can be used to discover a specific gene function when its role is already predicted, being analysed by the generated variants in comparison to the unchanged sequence. This targeted mutagenesis is often achieved by error-prone PCR (epPCR) [158], DNA shuffling [159], nicking mutagenesis [160,161], and PCR amplification using degenerate or random primers [161,162]. This targeted mutagenesis is a well-programmed approach, and there are several approaches to obtain it (including some of the above-mentioned techniques): homologous recombination [163], lambda Red recombineering [164], using site-specific recombinases [165], CRISPR systems [162,166], and bacterial retroelements [144].

2.3.1.1 Selection of phage deletion mutants through heat treatment and chelating agents

As described above, random mutagenesis generates several phage mutants and, among them, many are undesirable. From this pool, phages with the desired phenotype can be enriched by using a selective pressure, such as heat treatment [151,167–169]. This technique has been used to select naturally occurring phage mutants that have gene deletions thought to occur due to recombineering phenomena or incorrect replication across direct repeat sequences of 7-10 nucleotides [168,170,171]. The selection of genome-reduced phages using heat treatment resulted from early phage stability studies under high temperatures, being noticed that these heat-resistant phages usually have deletions in their sequences [154,167,168,172,173]. These deleted genes (0.5-3.5kb) are commonly referred to as non-essential genes for lytic development [169,171,173]. This phenomenon is observed to occur in a linear relationship between the heat inactivation curve of the mutant and the DNA content of that mutant [172,174]. Similar to temperature, chelating agents induce the same selection of a small proportion of the phage population (1-10% [171]) that are treatment-resistant and that contain gene deletions [172]. In the case of a chelating agent, it is proposed that chelating cations present in the phage surface destabilize the virion conformation [171].

The destabilization of the viral particle under high temperatures and chelating agent presence is a consequence of DNA loss from phage heads, being more pronounced when the two effects (ionic strength and temperature) are combined [169,171]. Initially, it was proposed to be related to smaller genomes exerting less pressure on the head structure [173,174]. Later, studies on viral metastability

have elucidated the balance between instability required for phage genome release upon cell infection and the stability of packaged genome in the capsid to survive under environmental conditions. In phages, this balance is ensured by a portal complex that forms a thin channel through the capsid wall and allows DNA release from phage capsid only after irreversible binding to the cell surface. Still, it should be noted that phage DNA is packed under high pressure (tens of atmospheres) provoked by DNA bending stress and forces provoked by electrostatic repulsion and hydration between neighbouring DNA helices [175]. Even though, in most environmental conditions, the viral capsid is highly resilient in maintaining packed DNA without premature injection. Nevertheless, thermostability is limited, and at high temperatures, the internal DNA pressure increases lead to DNA leakage from the capsid [175].

Several studies have proven that densely packed viruses also have higher internal pressures and that the balance between capsid stability and internal capsid pressure governs DNA injection. This DNA injection (which is spontaneous and irreversible) usually occurs at lower temperatures ($<70^{\circ}\text{C}$) than the major capsid proteins disassemble temperature and than viral DNA melting temperature [175]. Studies using DSC analysis (Differential scanning calorimetry) have shown that both enthalpy and temperature associated with DNA injection are shifted under chelating conditions (such as EDTA presence) [175]. These conditions reduce the temperature necessary for viral DNA injection and increase the injection enthalpy. By contrast, the presence of cations, such as Mg^{2+} , increases phage thermostability since when these ions permeate into phage capsid it stabilizes DNA and reduces repulsive forces, thereby reducing capsid internal pressure [174]. Curiously, it was also proven that genomes with smaller density (smaller length) have lower enthalpy (therefore smaller DNA pressure), resulting in a higher ability to resist to high temperatures [174,175]. Altogether, the elucidation of these molecular processes allows to explain the mechanism through which several authors have isolated phage mutants with deletions in their genomes after temperature and chelating agents' challenging. The advantage of this method is that it is easy to perform, fast and inexpensive, however, it is not suitable for targeted phage modifications. As such, the generation and selection of phage mutants is primarily used as a support to study the characterization of phage-host interactions. For instance, these mutants can help in the elucidation of essential phage genes, elucidation of phage receptor-receptor binding proteins for different bacterial hosts by the discovery of crucial residues that play a role in proteins functionality, validate genes annotation (usually based in homology with proteins present in databases), phage immunogenicity, phage efficiency, phage stability, creating detailed libraries that provide information for machine learning -based programs, reveal genes involved in phage lysogeny, insertion of reporter genes, understanding phage genes essentiality under different contexts, understanding phage evolutionary processes, and identification of phage regulation

circuits that can be further expanded to synthetic biology field. The knowledge generated through these approaches will help in the future to design novel phages with enhanced assets.

2.4 Phage-bacteria interactions

The relationship between phage and bacteria is complex, although the evolutionary dynamics of these two players is generally described as an arms-race hypothesis. In this hypothesis, the continuous bacterial exposure to phage predation leads to bacterial evolution toward avoiding phage predation. To acquire resistance to phages, bacteria use different defence mechanisms, such as CRISPR-Cas systems (prokaryote adaptive and inheritable immune system) [140], restriction-modification (R-M) systems [176], abortive infection mechanisms [177], cell surface modification, chemical defence [177], among others [178–180] (Figure 2.3A). By contrast, phages are intracellular parasites and are constantly pressured to assault bacterial cells and surpass cell defence mechanisms (e.g., by encoding anti-CRISPR/Cas proteins) [181] to successfully hijack bacterial metabolism to replicate itself and generate new virions that are released upon cell lysis (Figure 2.3B). This model appears to be straightforward since phages have an important role in the microbial communities by controlling their density, and in the case of prophages, it controls bacterial communication (through influencing the QS process) [182], bacterial resistance [183], bacterial escape from host immune system [184,185], virulence [186–188], evolution, genetic material exchange [189], biofilm formation [190], and superinfection exclusion mechanism [191] (Figure 2.3C). In addition, environmental conditions such as temperature [192], pH [193], nutrient availability, presence of host cells, and presence of polymicrobial communities can influence prey-predator interactions (Figure 2.3D). As such, phages can either be parasites or predators according to their life cycle, which is why only strict lytic phages can be used for phage therapy once temperate phages might be undesired for human application. Still, the interactions of lytic phages with their bacterial host remain poorly known and primarily limited to lab conditions, as the obtained results are usually dependent on the phage-bacteria pair.

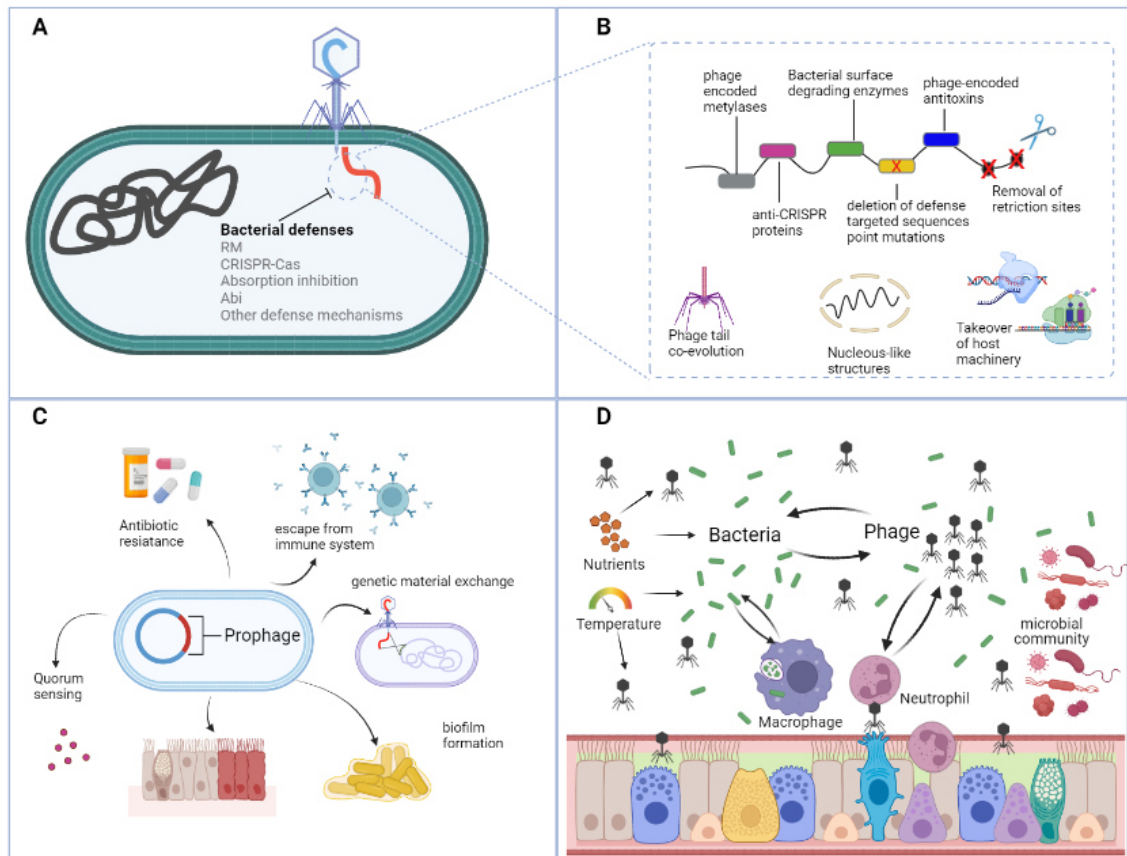


Figure 2.3- Overview of bacteria-phage interactions: A) bacteria defence mechanisms against phages; B) phage mechanisms that are used to bypass bacterial defences and to hijack its machinery; C) advantageous bacterial traits conferred by prophages; D) external factors that influence phage-bacteria interactions (nutrients, pH, temperature, immune system, antibiotics, host cells, and microbial communities, among others).

With the rise of the “omics era” new insights into phage biology had been acquired through the application of genomics, proteomics, transcriptomics, and metabolomic, which provided useful information about molecular mechanisms that occur during phage life cycle [194]. The discovery of phage-bacteria interactions covers the acquisition of information at each stage of the infection, such as the function of hypothetical phage ORFs known as “viral dark matter”, the bacterial responses during infection, bacterial fitness, and regulatory processes. The next sections will provide an overview on the current knowledge on lytic *Pseudomonas*-phages interactions with its host cell.

2.4.1 RNA sequencing (RNA-seq) applied to the discovery of phage-bacteria interactions

RNA sequencing (RNA-seq) is a high-throughput sequencing technique of the second generation that generates cDNA libraries from the RNA, allowing to capture the expression levels of multiple genes at once with high accuracy from a biological sample. It is a powerful tool for the global analysis of changes in transcription abundance [195,196], and usually it has been done through short-read sequencing Illumina [195–198]. The advantages of this method include high accuracy, sequencing of multiple samples, identification of gene isoforms, the discovery of novel transcripts, differentially expressed and regulated genes, absence of false-positives and cross-hybridization, precise definition of the RNA species at the single nucleotide level for both host and phage, probe-independence, mapping of the transcription start sites, and feasible *de novo* annotation [195,199]. Still, RNA-seq has a few limitations: bias in the cDNA library associated with the use of PCR for fragment amplification and the RT step, and loss of some RNA species and modifications. However, it should be noted that other approaches can be used to obtain RNA-seq libraries without PCR, such as the FRT-seq and DRS techniques on the Helicos platform, both producing short sequence reads [196,198]. Moreover, the RNA-seq requires ample storage space, high-level data management, powerful computational infrastructure, and there are bioinformatics challenges in the reconstruction of all genome transcription based on short reads [200]. Therefore, long reads are preferable for transcriptome analysis, which can be obtained by the TruSeq synthetic long reads (TSLR) technology recently introduced by Illumina [197], or alternatively, RNA can be directly sequenced using for example Nanopore sequencing. Still, RNA-seq is presently the most applied method to study gene expression levels and temporal transcriptional profiles during phage infection.

Using RNA-seq, valuable information about the global phage genome transcriptional scheme (early, middle, and late infection stage) has been provided [201], the diversity of phage-encoded modulators of gene regulation such as phage-encoded RNA polymerases (RNAP) [202], and comprehension of phage promoter sequences [203]. Nevertheless, a limited number of transcriptomic studies exist studying *P. aeruginosa*-phage interaction: using phage LUZ19 [204,205], phage ϕ KZ [206–208], PAK_P3 [209], phage C11 [210], PaP3 [211], phiYY [212], vB_PaeM_PA5oct [213], PaP1 [214], LUZ7 [215], PAK_P4 [216], and several phages simultaneously [217].

These studies provided greater insights about *Pseudomonas* phages, paving the way for new and more advanced studies that, for instance, try to improve the identification of phage-specific promoter and regulatory elements. For example, recently it was applied the dRNA-seq (differential RNA-seq) technique to study the transcriptome structure of the giant *Pseudomonas* phage ϕ KZ [208]. This resulted in a phage transcriptional map with detailed information about transcription starting sites and promoter elements on both the viral and the host genome in a single experiment. Alternatively, transcriptional

boundaries can be better identified by using a long-read sequencing platform. A recent study applied a long-read RNA sequencing that allows the sequencing of phage LUZ7 primary transcripts using the Nanopore platform, being this technique denominated ONT-cappable-seq [215]. In another interesting study, it was demonstrated that it is possible to unveil phage non-coding RNAs and capture the RNA-protein interactome during infection [207]. In the future, a new generation of RNA-seq techniques currently applied to bacteria to obtain high-resolution transcriptomes, phage RNAs interactome, RNA structure, and modifications, as well as transcriptomes at a single-cell level, might be adapted to phage research to better illuminate phage-host interactions [207,208,218–224].

2.4.2 Transcriptional mechanisms of tailed *Pseudomonas* phages

Similar to its host, *Pseudomonas* phages are very diverse, being these divided among the different families and grouped in specific clades according to its similarity. Some *Pseudomonas* phages are very well studied, and they've been used as models according to their genomic organization and transcriptional schemes [225]. The regulation of gene transcription is very important during phage lytic life cycle, and immediately after genome injection into bacteria cytoplasm, the recruitment of a transcriptional machinery is essential. In *Pseudomonas* phages, three main strategies can be adopted: a transcription where phage is completely dependent on host transcription machinery (phages do not encode RNA polymerase (RNAP)), other phages opt for semi-dependent strategy from host transcription machinery (phages only encode a virion RNA polymerase), and a strategy where phages are independent from host-transcription machinery (encode their own RNA-polymerase).

2.4.2.1 Transcription of phages that are dependent on host transcription apparatus

Phages that do not encode a phage RNAP (e.g., 14-1, LUZ24, YuA), usually harbour early promoters such as strong σ^{70} -like promoters, recognized by the host RNAP. After recognition, the host RNAP binds to the phage genome to start the transcription of early genes. The high affinity of host RNAP to phage promoters can be also mediated by phage proteins that bind to RNAP and alters its specificity (e.g., phage protein Alt from *E. coli* T4 phage) [226]. Next, early genes are transcribed to produce small proteins or factors that will modulate bacterial machinery and are essential during the viral cycle. This temporal transcription is mediated by phage proteins that bind to host RNAP, altering its specificity, resulting in a transition from early to middle and subsequently to the late infection phase. For example, the *P. aeruginosa* phage that uses this mechanism is phage 14-1, which encodes gp12 responsible for

binding to α subunit of host RNAP, leading to the transition of early phase to late phase transcription, or in the switch of host transcription to phage transcription [227].

2.4.2.2 Transcription mechanisms of *Pseudomonas* phages encoding a single-subunit RNAP (ssRNAP)

Another group of phages had evolved its transcriptional strategy to be only semi-dependent on host RNAP, which encode a ssRNAP. Normally these ssRNAP are employed for middle and late gene expression, like for example in LUZ19, LKA1, phiYY, and PAK_PA3 [227]. In this model, phage transcription is initiated by host RNAP that recognizes early gene promoters and the expression of early/middle genes is achieved by phage protein factors that will orchestrate the transition of genes transcription from host RNAP to viral RNAP. Therefore, at the end of the middle phase, the host RNAP is inhibited by phage proteins that bind to RNA β' subunit, for example, gp25.1 from LUZ19, and gp36 from LKA1 [206,228]. The viral RNAP is then able to recognize and bind late promoters to start the transcription of late phage genes. The host RNAP shutdown is an important step that allows the transition of early phase to later phase genes expression and besides being mediated by the binding of phage proteins, it can also be mediated by posttranslational modifications such as acetylation as observed in phage LUZ19. This phage encodes an acetyltransferase ('rac', gp28) that probably interacts with host proteases (ClpX and HslU) to cleave α RNAP subunit linker [205]. Besides, at the late stage of infection, it can be even expressed small RNAs that repress the host translational apparatus by binding/blocking bacterial ribosomes, as noticed in phage PAK_PA3 [209].

Inversely, for instance, *Pseudomonas* phage LUZ7 initially transcribe their early genes using a virion RNAP that is injected alongside with phage genome after adsorption, being late genes transcribed by the host RNAP. In the case of LUZ7, the protein responsible for binding the middle promoters and recruiting phage-encoded RNAPII to initiate transcription of middle genes was described recently [229].

2.4.2.3 Transcription mechanisms of *Pseudomonas* phages encoding multi-subunit cellular enzymes (msRNAPs)

A unique transcription strategy that is used by some phages is to be completely independent of host RNAP for its phage genes transcription. For example, ϕ KZ encodes two RNAP sets (β/β' subunits) needed for virion and non-virion phage-associated RNAPs that drive transcription of early and middle/late viral genes, respectively [208,230]. The virion RNAP (vRNAP) is transcribed from late genes and is packed with the phage genome at the end of phage infection. Upon infection, the phage genome and vRNAP are

injected, followed by the transcription of early genes is by vRNAP that is elongated through the middle genes, which contain the gene that encodes the second RNAP subunit. The second RNAP subunit is then responsible for the transcription of late genes including vRNAP [206].

2.4.3 *Pseudomonas* LUZ19 phage: A well-studied PhiKMV-like phage

Phage LUZ19 is a tailed phage with a strictly lytic life cycle. It has a dsDNA genome with a size of 43,548bp, with a GC-content of 62.2% and that has a genome structure similar to T7 coliphage [204,231]. This phage belongs *Autographiviridae* family and is a *Phikmvvirus* member [231,232], which is a genus containing many phages with high homology and that are widely spread over the world. Its burst size is about 122 particles/cell, completing an infection cycle within 24min [204,231]. In total, it has 54 open reading frames (Figure S2.1 and Table S2.1), all located in the sense strand, which can be divided in three main expression classes: early (gp.0.1 - gp17), middle (gp.18 - gp26), and late genes (gp27 - gp49) [231,232]. Most of the early genes have no assigned function and are suggested to be involved in most of the unknown mechanisms of host takeover during early infection [233]. Recently, gp4 (named by Qst) and gp11 have been demonstrated to be involved in *P. aeruginosa* PAO1 QS modulation, which seems to be essential for phage replication [234]. Contrary to early genes, the middle and late genes are well known, having roles associated with DNA metabolism [235], virion assembly, and host lysis, respectively. The bacterial receptor of phage LUZ19 is known to be type IV pili and its recognition is mediated by the tail fibers [231].

The transcription of *Phikmvvirus* phages seems to be largely performed by the host RNAP, which is recruited due to its affinity for strong phage promoters (that resemble host promoters) present at the beginning and the middle of phage early genes [204]. Host RNAP is switched for phage-encoded RNAP as a result of gp25.1 action, which is thought to bind to host RNAP β' subunit and inactivate it [228], and also through the action of gp28 ('Rac') that facilitates the α subunit cleavage [205]. Right after gp25.1 gene, is encoded the phage ssRNAP which executes the transcription of the following genes [204]. LUZ19 phage transcription is initiated from three strong promoters (that resemble with bacterial σ^{70} consensus sequence) at the genomic locations 913, 1147, and 1248 bp [204]. At 15min post-infection a second pattern is seen, with high expression of genes gp23, gp27, gp32, gp37, gp42, and gp47. Concerning to host transcripts, major differences were found in energy metabolism related genes (25 genes significantly regulated), being observed a downregulation in genes related with NADH dehydrogenase complex and cytochrome o ubiquinol oxidase subunits. Additionally, highly induced genes

were related with amino acid metabolism, and a downregulation in genes related with bacterial surface components was also observed (*wbp*, type IV pilus genes, and *arn* operons) [204].

According to highly conserved DNA replication genes, the *Phikmvvirus* members follow similar patterns of T7 coliphage replication. In short, DNA polymerase binds to a short RNA fragment synthesized by the host RNAP upon promotor recognition, allowing host DNA polymerase to manufacture the leading strand. The lagging strand synthesis are facilitated through the combined action of helicase/primase/single-strand binding protein that allows the replication in the opposite direction [236]. This bidirectional replication is hypothesized to start in the *ori*, which is thought to occur in the DNA polymerase gene [236]. Parallel with phage genome replication it was observed the breakdown of the host genome, most likely performed by 5' to 3' exonuclease (gene 22) and the type VII endonuclease (gene 23) to provide nucleotides essential for viral genome assembly [204]. For LUZ19 phage, the genome replication only occurs at 14 min post-infection, being this moment coincident with the start of host DNA degradation [204].

After DNA replication, the next phase is the production of structural proteins that constitute phage procapsids (major capsid proteins, scaffolding proteins, and head-tail connector proteins) and their assembly [237]. Although during infection the most abundant proteins seem to be gp10 (hypothetical protein), gp13, gp21, gp32 (major capsid protein), and gp41 (putative tail fiber) [204]. Next, phage DNA is packaged by the action of two terminase proteins (gp42-gp43) that identify the viral DNA concatemer, cut it into individual genomes and push it into the procapsid. Filled capsids are subsequently attached to phage tails to obtain mature viral particles, which are released upon cell lysis due to the combined activity of holin and an endolysin [238]. The former is responsible for causing small holes in the bacterial membrane, that allow endolysin access to the periplasm to exert its function of peptidoglycan degradation [239]. The fusion between inner and outer membranes is probably performed by the double action of Rz/Rz-like proteins (gp46-gp46.1), which are thought to encode spanins [238,240].

In the next sections interesting interactions captured during LUZ19 infection are highlighted as well as for other *Pseudomonas* phages, being these interactions powerful examples that drives the discovery of new mechanisms both from bacteria and phage sides.

2.4.4 Temporal gene expression of *Pseudomonas* phages

Besides the diversity of strategies employed by phages to use transcriptional machinery, the temporal expression schemes of *P. aeruginosa* phages genes appear to follow a consistent pattern: early (hypothetical proteins that are involved in host machinery reprogramming), middle (genes related to

nucleic acid metabolism), and late (phage structural proteins) genes expression. For example, phages, phages ϕ KZ [208], PAK_PA3 [209], PaP3 [211], and LUZ19 [204] are some of the *Pseudomonas* phages that seem to transcribe its genes in this order. A common feature observed during *Pseudomonas* phages infection is the tight control of host genes transcription, which is usually marked by the decrease in bacterial transcripts over time during infection. This was observed for phages PaP1 [214], PAK_P3 [209], PA5oct [213], LUZ19 [204], and ϕ KZ. Although, an exception was observed for the dsRNA phiYY phage that has a lesser proportion of phage reads over time compared to host reads, probably due to the small size of its genome, indicating a high dependency on host genes for its reproduction [212]. Apart from this last phage example, in general, phage diminishes the transcription of host “non-essential genes” by encoding proteins that bind to host RNAP and inhibit when it is necessary (e.g., gp25.1 from LUZ19, gp36 from LKA1, and other proteins from *Phikmvirus* phages) [205,228]. In addition to the generalized shutdown of host gene expression, the selective degradation of host transcripts is also a target process, where RNA degradosome (multicomplex that includes endoribonuclease RNase E) can be modulated. For example, Dip protein from ϕ KZ phage inhibits the degradation of phage transcripts by interaction with bacterial degradome, balancing therefore the host and phage transcripts turnover [241].

2.4.5 Manipulation of host transcription and replication machinery for new viral particles production

The mechanisms of how *P. aeruginosa* phages modulate the host transcriptional machinery are not yet fully understood and major efforts are yet to be done to deeply characterize the phages’ mode of action. In addition to characterizing the unknown ORFs from phage genomes, advances in the discovery of phage regulatory sequences also need to be done. Currently, efforts have been made to better annotate and predict transcription start sites (TSS), promotor sequences, antisense regulation mechanisms, and function of non-coding small RNAs, among others [207,208,213,215]. In addition to the lack of knowledge on underlying mechanisms behind phage transcriptional landscapes, there is also a big gap in understanding phage manipulation of the bacterial host towards a successful infection.

While phages shutdown “non-essential” bacterial functions, specific molecular mechanisms are recruited for the phage's benefit. According to phage replication needs, some metabolic pathways can be targeted including the upregulation of genes involved in transcription, translation, and post-translational modifications as verified for dsRNA phage phiYY, PAK_P3, PaP3, and LUZ19 [205,209,211,212]. According to the phage infection stage, the expression levels of those genes is tightly regulated, for instance, for phage PaP3, the downregulation of genes that encode ribosomal RNA and ribosomal

proteins is specifically achieved during late infection [211]. This orchestration of specific bacterial pathways at specific points of infection has been reported to be attained mainly through targeting bacterial global transcriptional regulators. An important target for phage PaP3 seems to be RpoS, a bacterial global response regulator that governs bacterial responses towards starvation, heat shock, osmolarity, antibiotic, and oxidative stress [242], since expression of phage gp70.1 leads to RpoS downregulation. In addition, other bacterial regulators like *dadA* and *fabA* are also downregulated [211].

A way to control regulators is to inhibit their expression, such as occurs during phiYY phage infection, where a cluster of transcriptional regulators is downregulated [212]. The manipulation of host regulators goes beyond the transcriptional level since these interactions also occur with functionally expressed host proteins. An example of this is the small protein SrpA encoded by *P. aeruginosa*, which is targeted by several phage proteins from phage K5. This host protein governs central cellular processes such as T3SS, chemotaxis, cell motility, cell shape, T6SS system, pyocyanin synthesis, biofilm formation, and it is an important regulator for phage DNA replication and phage transcription [243]. These examples prove the relevance of controlling bacterial global regulators to modulate bacterial metabolism. Recently, the protein product of an early gene from *Pseudomonas* LUZ24 (gp9, called “lgy”) was demonstrated to inhibit DNA gyrase subunit B of *P. aeruginosa*, impeding cell DNA replication, but the ultimate biological role during phage infection is unknown [244].

In some phages, modulation of host metabolism can be achieved by the expression of auxiliary metabolic genes (AMGs). These genes facilitate the acquisition of energy and metabolic products used by phages during infection. It's hypothesized that these genes have been acquired from the bacteria and improve phage infection. Phages that encode AMGs are PaP1 (thymidylate synthase, ORF 110) [214] and PAK_P3 (gp67, gp69, gp57, gp155 related to host mRNA and genome degradation; and gp151 encodes a cell wall hydrolase similar to the host) [209]. Thereby, the use of global bacterial regulators, phage AMGs, and phage regulatory proteins/factors seem to efficiently coordinate bacterial metabolism according to phage demands.

For some phages, it is possible to correlate the phage-targeted pathways with the stage of phage infection. For example, during the “middle infection stage”, a phase where the viral genome is replicated, it is common to see an upregulation of host genes associated with replication and nucleic acid metabolism, as well as phage genes necessary for viral DNA replication. This has been described for Pap3 infection where it is seen the co-expression of ORFs (ORF32, 39, and 40) involved in DNA replication with host genes *dnaN*, *gyrB*, *recF* related to DNA replication, recombination, modification, and repair [211]. Indeed, for phage genome replication the abundance of nucleotides is critical, which explains their

high abundance and the associated metabolites (e.g., pyrimidine) during the infection, as seen for *P. aeruginosa* infection with PAK_P3 [209]. Besides replication and transcription metabolisms, many other pathways seem to be significantly changed during phage predation, for example energy-associated metabolisms a common target which will be detailed in subsection 2.4.6.

2.4.6 Recruitment of viral building blocks by *P. aeruginosa* phages during infection

Bacterial metabolism control is crucial during viral infection since the phage needs to tightly manage energy consumption and 'phage building blocks' to successfully assemble new virions. Regarding this, the exploitation of amino acid metabolisms appears to be vital during the phage life cycle in order to obtain sufficient amino acids for phage proteins production. Indeed, this has been observed for several *P. aeruginosa* phages: ϕ KZ upregulates amino acid biosynthesis pathways (including arginine, leucine, glutathione, and phenylalanine biosynthesis pathways) [208] although, looking to a distinct phage, for instance PaP3, this pathway is regulated in a opposite manner [211]. Similarly, differences are found among the different phages, namely in the degradation of amino acids, where for dsRNA Phage phiYY [212] and PaP1 [214] genes related to the metabolism of amino acids are downregulated, but high coverage metabolism on *P. aeruginosa* infection with PAK_P3 verified that at late stage the metabolites from amino acid-related pathways were significantly reduced [209]. This possibly indicates that all recruited amino acids are being used for phage production. The recruitment/shutdown of amino acids metabolism is a common trait during phage infection, but so far it isn't understood the differences among the different phages, which might be related to specific strategies that vary according to the phase of infection, media conditions/bacterial fitness, or the phage itself.

Since many of the produced proteins during infection function as enzymes, the acquisition of associated co-factors is very important. One of the most important co-factors in *P. aeruginosa* is iron and during phage infection, it appears to be essential for phage replication. This is because iron is an essential cofactor of enzymes during new virions production (such as for DNA synthesis enzymes during viral DNA replication and ATP generation as energy for phage production) and even as an incorporated iron present in virions (in receptor binding proteins of tail fibers). Therefore, the upregulation of iron recruitment pathways is a must during infection. The interference with iron metabolisms is common in several *P. aeruginosa* phages: For example, ϕ KZ increases the expression of heme-binding bacterioferritin *bfrB* [204], as well as PAK_PA4 upregulates three operons related to the transport and production of siderophores (operons homologous to PAO1 *pchEFG*, *pchABCD*, *fpt* system, *pvdIJD*, *phuSTUVW* operon,

and *phuR* receptor gene, among others) [217]. In addition to iron, vitamins, such as vitamin B6 degradation can also be inhibited during phage infection [211].

2.4.7 Mechanisms of *P. aeruginosa* phages to obtain energy

Throughout the lytic cycle, phage impose a high energy burden on cells. As such, one of the main targets of bacterial metabolism is the pathways associated with energy production, even when it expresses phage encoded AMGs to facilitate this process. Intriguingly, it seems that phages tend to downregulate energy-associated pathways: downregulation of oxidative phosphorylation and nitrogen metabolism PaP1 [214]; in PaP3 there is a downregulation of NADH dehydrogenase encoding gene, F-type ATPase, succinate dehydrogenase, cytochrome c reductase, and cytochrome c oxidase [211]; downregulation of heme-copper oxidase family (F-type ATPase of complex V and the cytochrome bo3 ubiquinol oxidase) during ϕ KZ infection [208]; downregulation of pyruvate metabolism in early infected *P. aeruginosa*-PaP3 cells [211]; and downregulation of energy metabolism and carbon metabolism in phage phiYY [212]. On the other hand, small molecule transport involved genes *nark1*, *narH*, *narJ* seem to be upregulated in PaP1 [214]. In the case of PaP3 the secondary metabolite production seems to be unimportant for phage infection, as well as degradation of naphthalene [211]. Nevertheless, conclusions from these data should be carefully considered because in the majority of the transcriptomic/metabolic/proteomic studies, significant changes are obtained through the comparison of late-infected cultures to non-infected cultures, and consequently, these differences might be specific for the infection stage that is being evaluated.

Besides the acquisition of required concentrations of phage components, the recruitment of enzymes and metabolic pathways to produce new virions, phage needs also to control other bacterial aspects that might contribute to phage infection progress and if possible, increase the chances to spread and propagate in the neighbouring environments.

2.4.8 Phage mechanisms that influence lytic cycle success and viral progeny propagation

In the past decade, more attention has been given to the bacterial side during phage infection, since multiple underlying mechanisms of bacteria respond to phage infection and that some can lead to phage resistance. Functions related to bacterial phenotypes have then been of special interest such as changes in the expression of bacterial chemotaxis and motility. This was noted in PaP3-infected *P. aeruginosa* due to the downregulation of motility-related genes *pill* and *fliC* genes [242]. Also, it was observed the downregulation of motility-associated transcriptional regulators in ϕ KZ [208], and

chemotaxis repression in phages phiYY [212], PaP1 [214], and PaP3 [211]. This phenomenon has been described as a mechanism of phage to repress the dispersion of host cells to maintain cell proximity, and thus increase the success of infection of bacterial cells by the newly produced phages. This last explanation is consistent with the upregulation of putative adhesin CdrA involved in biofilm formation or cell aggregation seen in ϕ KZ, as a mechanism of enhancement of infection rate [208]. Similarly, during *P. aeruginosa* infection with phage PA5oct, the *napABCDEF* operon was upregulated (periplasmic nitrate reductase). This operon is involved in biofilm/cell aggregation [213]. Likewise, PA5oct infection leads to a strong upregulation of operon *psE-J* that is involved in the synthesis of *PsI* exopolysaccharide, associated with the formation of matrix. Once again, the induction of cell aggregation can be seen as a mechanism to increase phage spread through the neighbouring cells, but authors suggest that it can also be a mechanism of avoiding secondary infections by masking cell LPS [213]. Curiously, PB1-like *Pseudomonas* phages seem to encode peptides that interact with diguanylate cyclase YfiN, increasing c-di-GMP levels that affect bacterial morphology and induce small colony variants. These peptides ultimately lead to reduced cellular motility and increased biofilm formation [245]. Conversely, other studies reported the opposite, where they verified the inhibition of biofilm formation: during the expression of gp70.1 from phage PaP3 on *P. aeruginosa* biofilm was inhibited by the interaction of gp70.1 with biofilm regulator gene [242].

In terms of bacterial surface, different responses have been captured during phage infection. In the case of ϕ KZ-infected *P. aeruginosa* PAO1, there is a downregulation of LPS-associated genes (*wzx* and *wzy*) [191,208], which was hypothesized to be a defence mechanism to avoid cell infection with other phages. The last hypothesis is also suggested to explain dsRNA phage phiYY infection, which downregulates ADP-L-glycerol-D-mannoheptose epimerase (*rfaD*, PA3337), which is involved in LPS biosynthesis, limiting phage adsorption [212]. By contrast an upregulation of wall/LPS/capsule-related genes is observed in PaP3-infected bacteria [211]. In another study, precursor metabolites of the cell wall (UDP-N-acetyl-D-glucosamine or UDP-N-acetyl-D-galactosaminuronic acid) were founded to be increased after phage infection [209]. The contradiction among different studies is not yet understood and requires further studies to elucidate these tendencies.

2.4.9 Phage-encoded proteins to bypass host defences

Not of less importance are the strategies that phage employs to overcome bacterial phage-resistance mechanisms. To bypass bacterial surface modifications, it has been established that phages can evolve by changing their own receptor binding proteins (RBPs) [246]. After the adsorption process,

the phage injects its genome and rapidly needs to counter-interact the bacterial defences that recognize and degrade phage DNA (e.g., RM defence system and the CRISPR/Cas system). One way to resist to CRISPR immunity is by acquiring point mutations on the targeted DNA region [247], or the phage can encode anti-defence proteins that are expressed immediately after phage DNA injection such as anti-CRISPR/Cas proteins usually found in prophages and temperate phages [248–250]. In lytic phages, there are some examples of identified proteins that can avoid phage DNA degradation by interaction with host defences, such as LUZ24 gp4 (Mip, the MvaT inhibiting protein) that is highly expressed at an early stage and interacts with host MvaT by inhibiting its binding to AT-rich DNA (present on phage DNA) and consequent degradation [251]. MvaT/MvaU is also implicated in the *Pseudomonas* Pf4 prophage superinfection mechanism, being responsible for the inhibition of Pf4. Upon MvaT/MvaU loss, Pf4 can replicate and kill bacterial cells [252].

During infection, phage ϕ KZ also employs a mechanism that downregulates a type I restriction enzyme (PA2732) to avoid its DNA restriction, as well as upregulates antitoxin ParD to protect viral DNA from intracellular ParE toxins [208]. Similarly, dsRNA phage phiYY upregulates anti-oxidative stress genes (*ahpB* and *katB*) probably to protect against genomic RNA degradation which is more sensitive to degradation rather than DNA genomes [212].

2.4.10 Other bacterial responses toward phage infection

Curiously, another mechanism often induced during *P. aeruginosa* phages infection (PA5oct LUZ19, PEV2, PAK_P3, and PAK_P4) is the transcription of prophage-associated genes contained within the host genome [204,209,213,217]. Their transcription is a direct consequence of a global bacterial stress that is induced by phage infection. This increase in bacterial stress functions as a signal for prophage survival which is induced under these conditions. The relevance of prophage induction during lytic *Pseudomonas* phages infection remains to be well elucidated. This phenomenon should be carefully addressed from a phage therapy context, since the release of temperate phages can have a negative impact. For example, *Pseudomonas* prophages have been described to positively impact biofilm formation [253], virulence [254,255], interspecies competition [253,256], and type IV pili inhibition [191]. Also, “morons” (prophage sequences) can increase bacterial lysogen fitness against further lytic phage infections [257]. A recent study revealed that wounds from mice, pigs, and humans colonized with *P. aeruginosa* harbouring pf prophage have impaired healing comparing with wounds colonized with bacteria that lack Pf prophage [258].

QS is also one way for bacteria to defend the population from phage attack [259]. Nevertheless, phage-bacteria interactions can shape PQS-mediated virulence of *P. aeruginosa* [259]. For some lytic *P. aeruginosa* phages, PQS metabolites were found to be abundant in infected cultures [225] and were proposed to be a host-mediated stress response towards phage infection. But for some phages (PEV2, ϕ KZ, and LUZ19) this phenomenon isn't valid, indicating that these phages might evolve mechanisms to block PQS synthesis [217,225,260]. This might be the case for ϕ KZ that during its infection downregulates PQS transcriptional regulators to decrease bacterial virulence [208]. For LUZ19, the role of a phage-encoded protein (Qst, 'quorum sensing targeting protein) was described, and induces decreased PQS levels by direct interaction of Qst with PqsD (a host enzyme from quinolone signal biosynthesis pathway), and curiously phage infection is limited in the absence of this enzyme. Also, the lysogenic phage DMS3 encodes in its genome a QS anti-activator protein (Aqs1) that inhibits the master regulator of QS LasR and inhibits type IV pilus assembly (blocking superinfection) [261].

During the later stages of infection, bacterial stress responses have been captured, such as the ones associated with heat shock response [262,263], oxidative stress, and proteolysis [264,265]. The upregulation of SOS and DNA repair systems during phage infection was proposed to be a result of phage targeting by host CRISPR immunity [266]. However, on the other hand, it was reported that during ϕ YY infection, genes that encode antioxidant products are upregulated, which was attributed to a phage mechanism that induces genes that minimize host stress responses during infection [212]. Bacterial responses to phage infection are highly dependent on the phage-host pair and vary according to the viral cycle phase.

2.4.11 *P. aeruginosa* virulence manipulation by lytic phages

In several transcriptome studies the impact of phage infection on bacterial virulence has been addressed to guarantee the safety of phage therapy. This is a relevant question from a phage therapy context because it is known that phage-resistant bacteria have reduced virulence [267], which aids in the efficacy of antimicrobials and patients' immune systems [268]. From a molecular point of view, it was observed that host-associated virulence genes are downregulated upon phage infection. For example, T3SS system, needle protein (*pscF*), ATPase (PA1697), the virulence regulator *VqsR*, *ExsA* the transcriptional activator of the *P. aeruginosa* T3SS were all downregulated in *P. aeruginosa* infection with ϕ KZ phage [208]. In PaP1-infected *P. aeruginosa*, authors also reported the repression of a two-component system, as well as, the downregulation of operon *BetAB* involved in glycine betaine production, resulting in the inhibition of choline–glycine betaine pathway, which is highly toxic for *P. aeruginosa* [214].

In addition, PaP3 phage also encodes a protein that globally represses bacterial virulence by interacting with transcriptional regulators *argR* and *vqsR* related to QS system and virulence respectively. This reduction in virulence mediated by PaP3 ORF was demonstrated in mice survival assays [242]. Likewise, a small protein SrpA from *P. aeruginosa* was identified as essential for phage K5 infection. SrpA is a modulator of several bacterial mechanisms such as chemotaxis, motility, biofilm, pyocyanin syntheses, protein secretion, and virulence in the infection model of *Caenorhabditis Elegans* [243].

Many mechanisms of phage-bacteria interactions have yet to be discovered and with the application of integrative omics technologies, the knowledge gap in the phage field could be resolved. As a first step towards the implementation of these global omics approaches in phage research, obtaining high-resolution transcription maps is crucial to fully elucidate the gene expression profile phage-host pair and elucidate regulatory processes at a molecular level [194].

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2.6 Supplementary data

Table S2.1- Detailed Genome annotation of *Pseudomonas* LUZ19 phage. Location, name, and size of each feature product are detailed.

Location Start	Location end	Feature type	Name	Product	Size (bp)
1	472	repeat region	-	-	472
913	948	regulatory	promoter	sigma70 consensus sequence	36
982	1,017	regulatory	promoter	sigma70 consensus sequence	36
1,147	1,182	regulatory	promoter	sigma70 consensus sequence	36
1,616	1,744	gene	gp0.1	hypothetical protein	129
1,757	2,041	gene	gp1	hypothetical protein	285
2,041	2,268	gene	gp2	hypothetical protein	228
2,279	2,818	gene	gp3	hypothetical protein	540
3,130	3,149	regulatory	promoter	phage-specific promoter sequence	20
3,186	3,554	gene	gp4	hypothetical protein	369
3,541	3,765	gene	gp5	hypothetical protein	225
3,944	4,204	gene	gp6	hypothetical protein	261
4,204	4,494	gene	gp7	hypothetical protein	291
4,494	4,724	gene	gp8	hypothetical protein	231
4,729	5,016	gene	gp9	hypothetical protein	288
5,095	5,511	gene	gp10	hypothetical protein	417
5,580	5,939	gene	gp11	hypothetical protein	360
5,942	6,751	gene	gp12	hypothetical protein	810
6,753	6,772	regulatory	promoter	phage-specific promoter sequence	20
6,831	7,250	gene	gp12.1	hypothetical protein	420
7,276	7,563	gene	gp13	hypothetical protein	288
7,553	7,681	gene	gp13.1	hypothetical protein	129
7,749	8,573	gene	gp14	primase	825
8,617	9,810	gene	gp15	AAA family ATPase	1,194
9,800	10,420	gene	gp16	hypothetical protein	621
10,420	11,367	gene	PPLUZ19_PPL UZ19_gp20	DNA Ligase	948
11,364	11,684	gene	gp18	hypothetical protein	321
11,681	14,104	gene	gp19	DNA polymerase	2,424
14,101	14,412	gene	gp20	hypothetical protein	312
14,413	14,432	regulatory	promoter	phage-specific promoter sequence	20
14,467	15,516	gene	gp21	hypothetical protein	1,050
15,516	16,457	gene	gp22	5'-3' exonuclease	942

16,447	16,887	gene	gp23	endonuclease type 7	441
16,884	17,930	gene	gp24	hypothetical protein	1,047
17,940	18,311	gene	gp25	hypothetical protein	372
18,304	18,654	gene	gp25.1	hypothetical protein	351
18,663	21,110	gene	gp26	phage-specific RNA polymerase	2,448
21,283	21,534	gene	gp27	hypothetical protein	252
21,534	22,007	gene	gp28	GNAT family N-acetyltransferase	474
21,973	22,248	gene	gp29	hypothetical protein	276
22,260	23,792	gene	gp30	head-tail connector protein	1,533
23,796	24,764	gene	gp31	scaffolding protein	969
24,765	24,784	regulatory	promoter	phage-specific promoter sequence	20
24,817	25,824	gene	gp32	major capsid protein	1,008
25,921	26,475	gene	gp33	tail tubular protein A	555
26,478	28,958	gene	gp34	tail tubular protein B	2,481
28,958	29,503	gene	gp35	internal virion protein	546
29,503	32,199	gene	gp36	internal virion protein	2,697
32,203	36,216	gene	gp37	internal virion protein	4,014
36,218	36,973	gene	gp38	phage particle protein	756
36,973	37,431	gene	gp39	tail fibre protein	459
37,424	38,329	gene	gp40	tail fibre protein	906
38,333	38,938	gene	gp41	tail fibre protein	606
38,938	39,243	gene	gp42	terminase small subunit	306
39,253	41,058	gene	gp43	terminase large subunit	1,806
41,055	41,255	gene	gp44	phage holin	201
41,252	41,734	gene	gp45	lysozyme	483
41,692	42,021	gene	gp46	Rz protein	330
41,909	42,109	gene	gp46.1	Rz1 protein	201
42,111	42,425	gene	gp47	phage particle protein	315
42,473	42,718	gene	gp48	phage particle protein	246
42,728	42,943	gene	gp49	hypothetical protein	216
43,077	43,548	repeat region	-	-	472



Figure S2.1- *Pseudomonas* LUZ19 phage genome. Coding sequences are identified in yellow, regulatory sequences in green, and repeat regions in orange. Image was performed in Geneious Prime.

CHAPTER III: TRANSCRIPTOMICS OF *PSEUDOMONAS* LUZ19 PHAGE INFECTED BACTERIA UNDER *IN VITRO* CONDITIONS

This chapter is based on following scientific article:

- Brandão, A., Pires, D.P., Coppens, L., Voet, M., Lavigne, R., and Azeredo, J. (2021) Differential transcription profiling of the phage LUZ19 infection process in different growth media. *RNA Biol* 00: 1–13.

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Abstract

RNA-seq of phage-infected bacterial cultures offers a snapshot of transcriptional events occurring during the infection process, providing insights into the phage transcriptional organization as well as the bacterial response. To better mimic real environmental contexts, it was performed RNA-seq of *P. aeruginosa* PAO1 cultures infected with phage LUZ19 in MCCM medium to better simulate a phage therapy event and the data were compared to LB medium.

Regardless of the media, phage LUZ19 induces significant transcriptional changes in the bacterial host over time, particularly during early infection ($t=5\text{min}$) and gradually shutdown bacterial transcription. In a common response in both media, 56 *P. aeruginosa* PAO1 genes are differentially transcribed and clustered into several functional categories such as metabolism, translation and transcription. The data allowed to tease apart a medium-specific response during infection from the identified infection-associated responses. This reinforces the concept that phages overtake bacterial transcriptome in a strict manner to gain control of the bacterial machinery and reallocate resources for infection, in this case overcoming the nutritional limitations imposed by MCCM medium. From a phage therapy perspective, this study contributes towards a better understanding of phage-host interaction in human physiological conditions and demonstrates the versatility of phage LUZ19 to adapt to different environments.

3.1. Introduction

Phages are highly diverse viruses that specifically infect bacteria and are ubiquitous in nature. These viruses have been intensively studied and have highly contributed to the progress of various research fields [1]. Besides the major advances in phage research, such as high-throughput genome sequencing that contributed to better understand the enormous molecular diversity of phages, these viruses still represent the “dark matter” of biological world [2]. The principal knowledge gaps in phage biology are linked to the identification and comprehension of the interactions between phage and bacteria during the viral infection cycle, at a systems biology level [3,4]. Understanding these interactions can reveal how phage modulate the bacterial metabolism and inhibit bacterial defence mechanisms to establish an effective infection [5]. Several omics approaches have contributed to the discovery of new phage-host interactions and mechanisms. Among these, transcriptomics analysis using RNA-seq is emerging as a valuable tool to study phage and bacteria gene expression during specific steps of infection, enabling the discovery of new genes and their functions, and the identification of regulatory RNAs [6]. The first studies have applied RNA-seq to unveil interactions between phage-bacteria pairs [6–15]. These works are focused on the study of phage and bacterial transcriptome in standard growth media at different stages of infection (usually early, middle, and late infection). Despite providing a deeper knowledge on phage-host interactions, these studies do not provide insights about the molecular mechanisms of interaction that occur when bacteria are exposed to non-optimal growth conditions. Therefore, increasing the complexity of these assays by introducing conditions that better mimic the real environments where phage and bacteria interact, might clarify if there are novel phage-host interactions to be discovered. This is particularly important in a context of phage therapy, where phages interact with bacteria in human physiological conditions, which are far from the ideal growth environments provided by standard optimal growth media.

Here it was studied the transcriptional profiles of *P. aeruginosa* PAO1 cultures infected with phage LUZ19 under a standard optimal growth medium versus a MCCM medium. LUZ19 phage is a well-characterized virulent podovirus that targets *P. aeruginosa* [16,17], and a candidate phage for therapeutic purposes. Previous studies of LUZ19 transcriptome revealed a clear temporal expression pattern within the phage, as well as a major impact on transcription of host metabolic genes and on type IV pilus expression regulation [16].

In this chapter, it is investigated the effect of different media in both phage and bacteria transcriptome at different stages of infection. Using RNA-seq, it is aimed to understand the gene

expression patterns associated with the infection process in standard LB medium versus MCCM medium. This work constitutes a first step towards a better understanding of phage-host interactions under conditions that better mimic the real *P. aeruginosa* infections in human patients.

3.2. Materials and Methods

3.2.1. Bacterial strains, phage propagation and growth conditions

The reference strain *P. aeruginosa* PAO1 (DSM22644) from The German Collection of Microorganisms and Cell Cultures was used in all experiments and was grown at 37°C on Lysogeny Broth (LB) or LB agar (LB with 1.2% (w/v) of agar). Specific experiments were performed using Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM:F12) supplemented with 2% (v/v) of Ultrosor-G Serum Substitute Medium. This medium supports the growth of human airway epithelial cells and was chosen to mimic human bronchial physiological conditions, referred as MCCM [18]. *Pseudomonas* phage LUZ19 [17] was propagated on *P. aeruginosa* PAO1 cells by standard soft agar overlay, followed by PEG8000 precipitation and stored in phage buffer pH 7.5 (10mM Tris-HCl, 10mM MgSO₄, 150mM NaCl) at 4°C [19]. Phage titration was performed using the double-agar layer [20].

One-step Growth Curves (OSGC) were established on either LB or MCCM media. Briefly, cultures grown until OD_{600nm}=0.3 were infected with phage at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C with 120rpm for 5min. The mixture was centrifuged (7,000×g at 4°C, 5min) to remove free phages and then the pellet was resuspended in fresh medium. Phage titer was quantified at 5min intervals over a period of 30min. The PFU/cell was accessed through the ratio between the PFU/mL in each time point and the initial PFU/mL (t=0min).

3.2.2. Synchronized infection assays

P. aeruginosa PAO1 overnight cultures were diluted 1:100 in 25mL of either fresh LB or MCCM medium and grown to the early-exponential phase (OD_{600nm}=0.3, corresponding to approximately 2×10⁸ CFU/mL). At this point, the number of colony forming units of the culture (CFU/mL) were counted by plating serial dilutions in LB plates [21] and a sample of 4.5mL of culture was taken simultaneously (t=0min). This sample was immediately mixed with 1:10 vol of an ice-cold stop solution (1:10 buffered phenol, 9:10 absolute EtOH) and kept on ice to stabilize RNA. The remaining culture (≈20mL) was transferred to a pre-warmed Erlenmeyer flask and infected with phage LUZ19 at a MOI = 75 (to ensure synchronous infection of the culture) and incubated at 37°C and 120rpm.

At t=5 (early infection), t=10 (middle infection) and t=15 (late infection) minutes post-infection, 4.5mL of culture was mixed with chilled stop solution and placed on ice. Immediately after, all collected samples were frozen at -80°C to block RNA transcription and degradation until sample processing.

Biologically independent infection experiments, showing a CFU reduction of at least 95% within 5min post-infection (only 5% bacterial culture survival to LUZ19 infection) were considered as synchronized infection. Triplicate samples from synchronized infections were used for total RNA extraction.

3.2.3. Total RNA extraction, quantification, quality assessment and rRNA depletion

Samples from synchronized assays were thawed on ice and centrifuged (3,345×g, 20min, 4°C). Total RNA was extracted from cell pellets using the Purelink RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Subsequently, each sample was treated with TURBO DNase (Invitrogen) at 37°C to remove genomic DNA (gDNA) and then co-precipitated overnight at -20°C with 1:10 (v/v) NaOAc and 2.5 vol of absolute EtOH.

Precipitated RNA samples were centrifuged (16,000×g, 4°C, 1h) and pellets were washed twice with 70% (v/v) EtOH. The air-dried RNA pellets were dissolved in 60µl of DNase and RNase free water (Invitrogen). Total RNA concentration and purity were measured on a Nanodrop 2000 Spectrophotometer (Thermo Scientific, MA, USA) and on a Qubit 4 Fluorometer (ThermoFisher Scientific). In addition, the absence of gDNA contamination was confirmed by PCR using specific primers for both phage LUZ19 (Forward: 5'-ccgacaagatcgctccgcatgc-3', Reverse: 5'-ggctgaagctacgcttgcc-3') and its host (Forward: 5'-cggtcggaatctctacgcacc-3', Reverse: 5'-cgacgccaacaacgccacg-3').

To assess RNA integrity, samples were analysed on a Bioanalyzer (Agilent, Santa Clara, California, US) with a RNA 6000 Nano Kit (Agilent) according to manufacturer's instructions. Samples with good RNA integrity (RNA integrity number (RIN) > 8) were treated with RiboMinus™ Transcriptome Isolation Kit (bacteria) (Invitrogen) to deplete ribosomal RNA (rRNA), according to the manufacturer's instructions.

3.2.4. cDNA libraries preparation and sequencing

Depleted rRNA samples were precipitated and washed as mentioned previously and resuspended in 18-19µL of Fragment Prime Finish mix from TruSeq Stranded mRNA Library Prep (Illumina). Successful rRNA depletion was verified on a Bioanalyzer and the final concentrations were determined using the Qubit 4 Fluorometer (Invitrogen).

rRNA-depleted samples were processed immediately, according to TruSeq Stranded mRNA Library Prep recommendations. In the end, cDNA libraries were evaluated on a Bioanalyzer (Agilent) to confirm average fragments between the range of 200-300bp and final concentrations were measured on a Qubit. The libraries' normalization and pooling were performed according to MiniSeq System Denature and Dilute Libraries Guide: protocol A from Illumina. For each run, a library of three samples with equimolar amounts was combined with PhiX control (spike-in of 0.5-2%) and paired end sequenced (2×75bp) on an in-house Illumina Miniseq sequencer.

3.2.5. Data analysis

The quality of cDNA reads was analysed using FastQC (version 0.11.2) [22]. Paired-end reads were aligned to the LUZ19 and *P. aeruginosa* PAO1 reference genomes (NC_010326.1 and NC_002516.2) using Bowtie 2 [23]. Next, the aligned reads were assigned to gene features using featureCounts [24]. Reads that mapped to rRNA (60-80%) were removed *in silico*. Subsequently, counts were first normalized by gene length and then by the number of non-ribosomal reads in each sample to obtain RPKM values. Samples variance was analysed using Principal Component Analysis (PCA) in R and outliers were excluded from transcriptional analysis. Therefore, samples from assay MCCM_3 were discarded while samples from two independent assays in MCCM and samples from three independent assays for LB medium were considered for further analysis. Heat map of *P. aeruginosa* PAO1 genes was also built (Figure S3.3). Differential gene expression analysis was performed using negative binomial distribution test from DESeq Bioconductor package in R [25]. ANOVA test was performed to identify the most significantly up or downregulated genes. The p-values were adjusted using the “BH” method [26], which is the standard method built into the DESeq package and are referred to as “padj”. Raw data and processed data are available under the accession GSE162278.

3.3. Results

3.3.1. Phage transcripts gradually take-over the total transcriptome in both LB and MCCM media

Unveiling phage-host interactions depends on the knowledge acquired during infection process in a one-step infection cycle, as previously optimized [16]. To get a global overview of phage infection at the transcriptional level, it was tracked the non-rRNA transcripts as a function of time during the infection, both for *P. aeruginosa* PAO1 grown in LB and in MCCM medium, as illustrated in Figure 3.1. Before

infection (t=0min), all transcripts logically map to the host genome. Five minutes post infection, this percentage varies between 60% and 85%. However, at the middle (t=10min) and late (t=15min) time points, the total number of host reads are reduced to 20-25% and 3-8%, respectively. At this late stage of infection, the reads that map to phage genome dominate the sample in both LB and MCCM cultures.

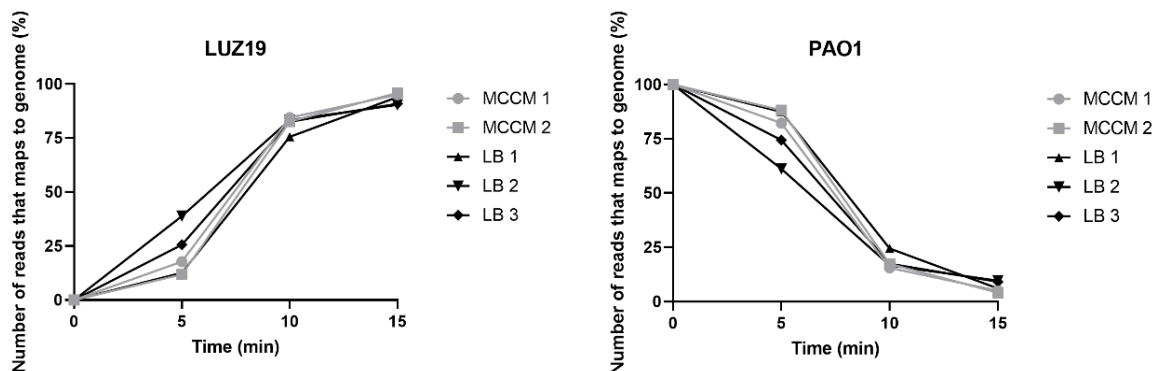


Figure 3.1- Percentage of reads that map to the genomes of phage LUZ19 and *P. aeruginosa* PAO1 for individual replicate samples during synchronized infections in LB or MCCM medium.

Comparing the phage transcriptional maps for time points 5, 10 and 15min in both media, expression patterns of the individual time points appear to correlate well to each other (Figure 3.2). Furthermore, these transcriptional maps correlate well with previous data and imply a logical progression from early (host adaptation), to middle (DNA replication) and late (structural and lysis proteins) expression for this phage [16]. However, it should be noted that a slight delay in phage transcription was observed for the MCCM-grown, infected cultures. Nevertheless, this minor discrepancy does not impact the results and interpretations outlined in the next sections, in fact OSGC demonstrated that the infection occurs equally in both medium in terms of latent period (Figure S3.1), but there was a visible change in the burst size when it is used different media. Zooming in on the expression levels of individual phage genes allows to identify differentially expressed genes ($p\text{-value} < 0.05$) at early, middle and late stages of infection (comparing with uninfected cultures) for both growth media conditions. Overall, phage gene expression levels do not differ between the LB and MCCM grown bacterial cultures.

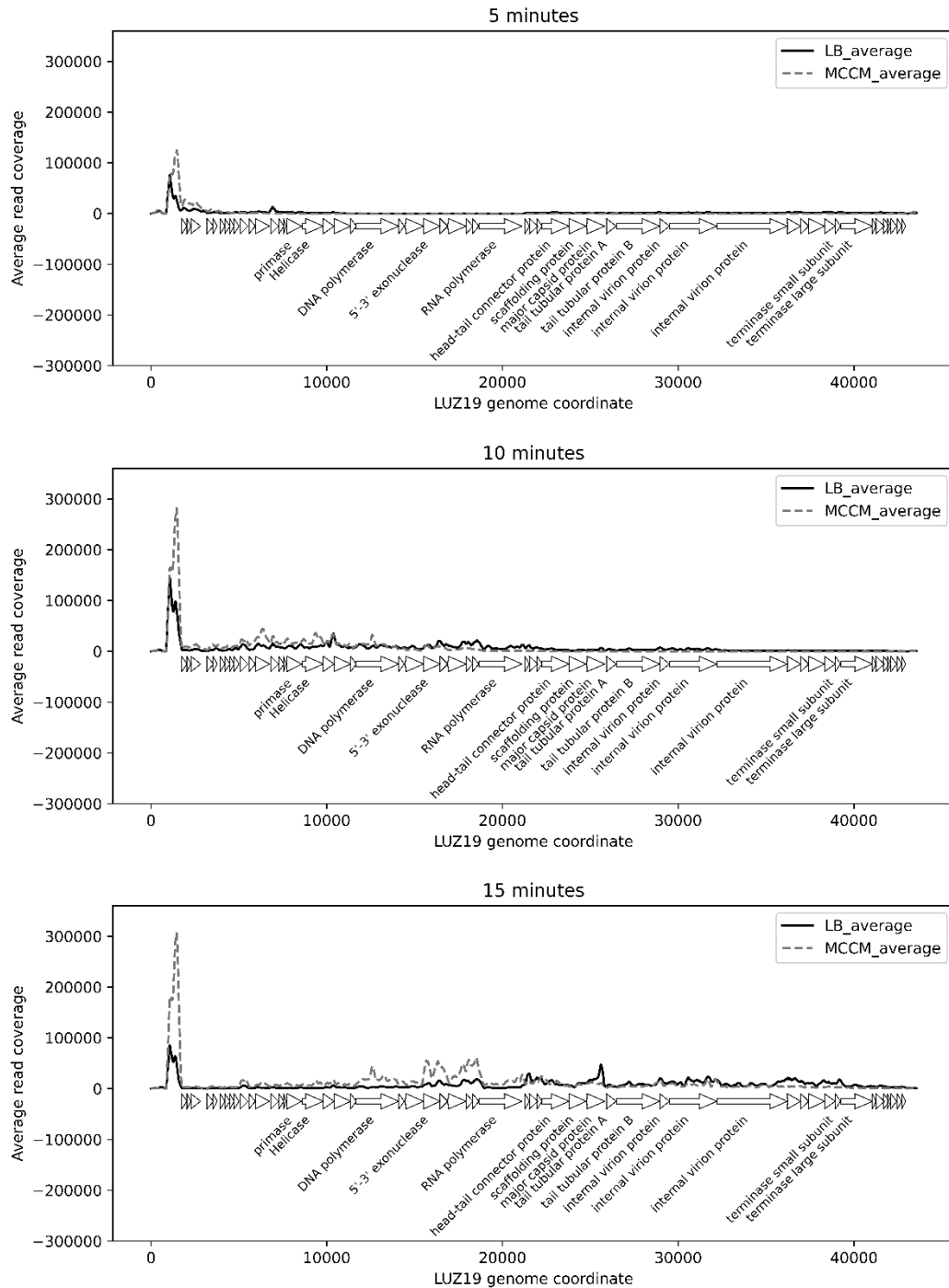


Figure 3.2- Distribution of cDNA reads over phage genome at early (t=5min), middle (t=10min) and late (t=15min) infection when LB or MCCM media were used.

3.3.2. *P. aeruginosa* PAO1 transcription is markedly impacted by the growth media used

To better understand the influence of growth media on phage infection, the medium impact on the bacterial transcriptome (without phage infection) was analysed as a first step. The indication that

medium has a strong influence on host behaviour was further confirmed by comparing the transcriptome of uninfected-PAO1 cultures (t=0min) grown in MCCM versus LB. Differentially expressed genes were considered to have a $\text{Log}_2\text{Fold} \geq 2$ (upregulated genes) or ≤ -2 (downregulated genes), and a $\text{padj} \leq 0.05$. A total of 383 and 239 differentially expressed genes were found to be up and downregulated in MCCM medium comparing to LB medium, respectively. From a general perspective, the majority of the differentially expressed genes have no assigned category or unknown function (category S), based in Clusters of Orthologous Groups (COGs) categories. However, there is also a high number of genes differentially expressed which belong to categories related to metabolism (Figure 3.3). In addition, major differences associated with transcription [category K] and cell wall/membrane/envelope biogenesis [category M] functions are also influenced by the used medium. Interestingly, among the upregulated genes in MCCM vs. LB, 74 are related to iron acquisition, transport, storage and virulence factors such as phenazines and pyoverdine (Table S3.1). Many of these genes are regulated by the iron/heme/siderophores levels and are induced under starvation conditions. The fact that these genes are upregulated in MCCM medium compared to LB medium indicates that bacteria need to acquire these substrates when growing in MCCM. In contrast, in LB medium these genes are downregulated, which shows the absence of iron starvation. In addition, a total of 69 genes (Table S3.2) involved in secretion system (type I, II, III and IV), two-component system, biofilm formation, QS, multidrug resistance and virulence factors, are also upregulated in MCCM medium relative to LB medium.

Overall, the large diversity of genes that are differentially transcribed in MCCM compared to LB medium proves that the composition of the media drastically influences the *P. aeruginosa* pathogenicity and virulence in addition to the bacterial metabolism.

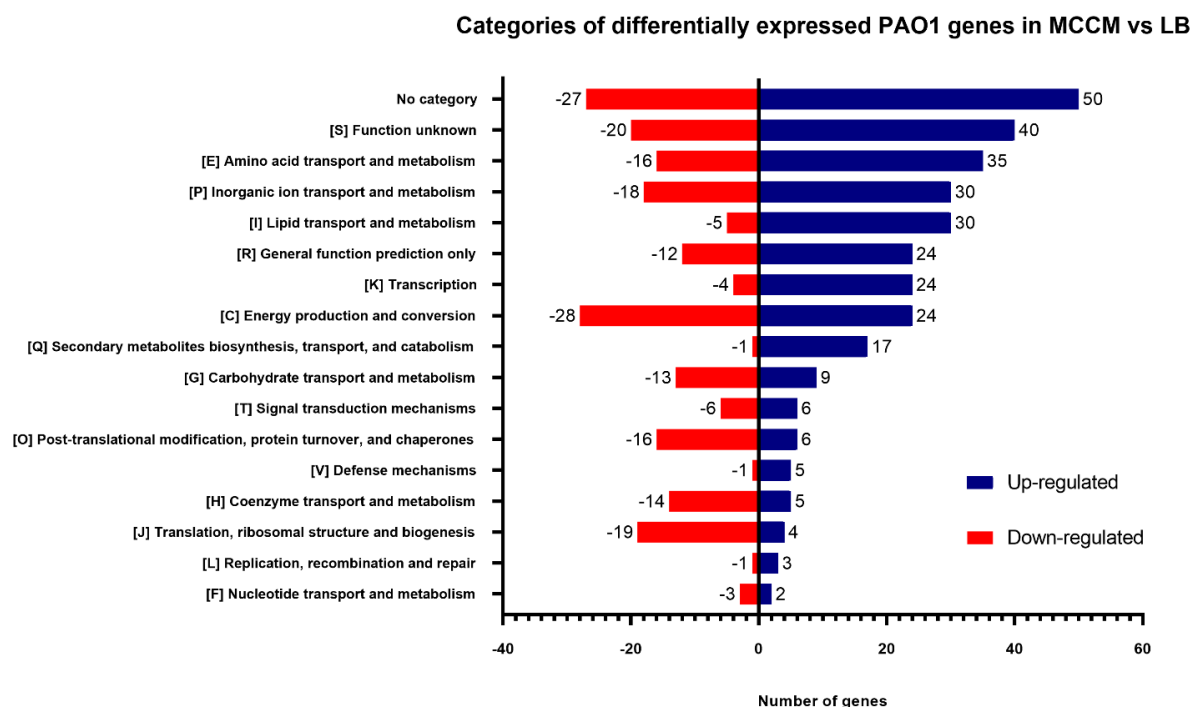


Figure 3.3- Number of up and downregulated genes per COG category from non-infected bacterial cultures. DE are relative to MCCM versus LB.

3.3.3. Elucidating the bacterial response to phage infection

A PCA enabled the identification of the parameters that most influence the variation of transcriptome profiles of *P. aeruginosa* PAO1 infected by LUZ19 in LB and MCCM culture media. The obtained PCA (Figure 3.4), suggests that the difference between the samples is explained by two major dimensions: in the first-dimension the variance is attributable to the different stages of phage infection (66.6%), whereas the variance in second dimension is caused by the medium (14.2%). In the first dimension of the PCA analysis, the transcriptomes from 5min post-infection are very different from those acquired at 10 and 15min. In the second-dimension main differences are observed between media only at 0 and 5min, but at 10 and 15min post-infection the transcriptomes for both media converged.

In the next section, the principal changes in terms of energy production, two-component regulatory systems and replication-, transcription- and translation- processes will be highlighted.

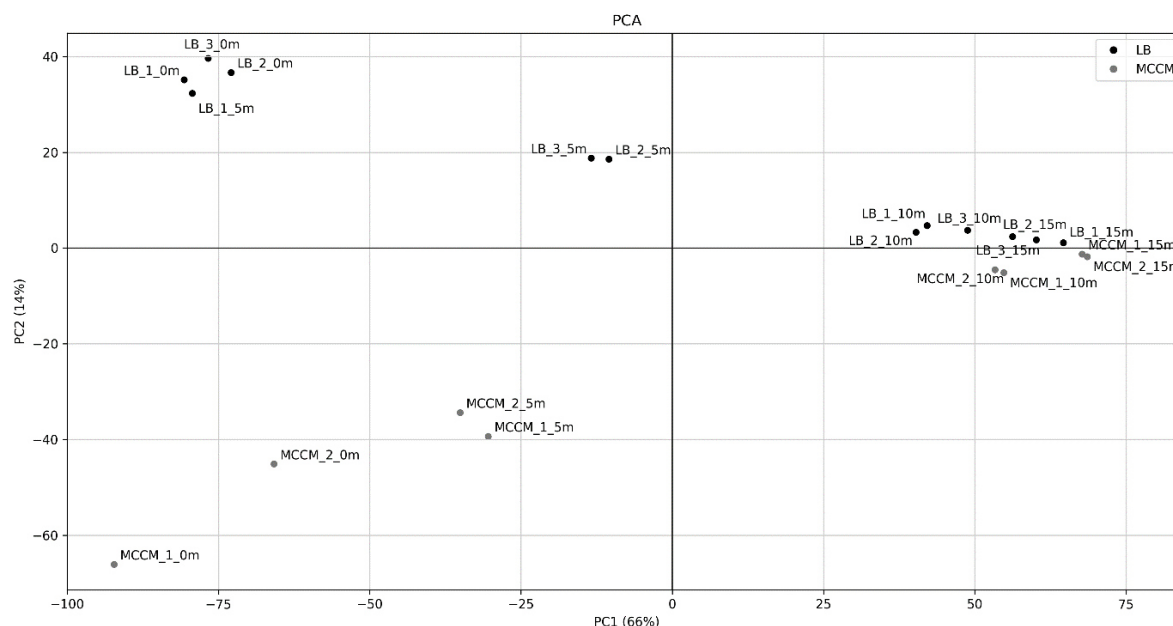


Figure 3.4- Principal Component Analysis (PCA) of the transcriptome obtained from samples acquired during different time points of synchronized assays when LB or MCCM media were used.

3.3.4. Phage infection strongly impacts bacteria transcriptome regardless of the growth conditions

Since the major differences between the samples acquired from phage-infected bacteria in different media occur at 5min post-infection, the bacterial differential expression genes (DEG) were evaluated in detail at this time point, relative to uninfected cultures. At early infection, all differentially expressed PAO1 genes relative to uninfected cultures, in both media, were selected. Among them, 56 genes (Figure S3.2) are commonly differentially expressed in both media (Table 3.1 and Table 3.2), indicating that this is a specific response to phage infection, regardless of the medium. Therefore, the function of these 56 genes were analysed in detail in the next subsections.

3.3.4.1. Energy production is strongly promoted at early infection period

The majority of genes referred above, are involved in metabolic pathways as summarized and contextualized in Figure 3.5. More specifically, these genes are associated with energy production within the TCA cycle or transport of its intermediates including: C4-dicarboxylates transport, Riboflavin biosynthesis, FAD synthesis, amino acids transport and metabolism, electron chain transport, among others. In this study, genes that encode C4-dicarboxylates transporter components (*dctMQP* genes) are

clearly upregulated in both media compared to uninfected cultures, indicating a need for these substrates during phage infection. In addition to upregulation of genes involved in the transport and metabolism of carbon sources, the production of riboflavin and its derivatives (FAD and FMN) are also upregulated at an early infection stage in both media. Another upregulated genes involved in energy acquisition are: the gene that encodes for formate dehydrogenase-N β -subunit (*fdnH*), the genes involved in putrescine conversion into succinate (*spuC*, *davD*, *davT*), and genes involved in putrescine and spermidine transporter (*spuF*).

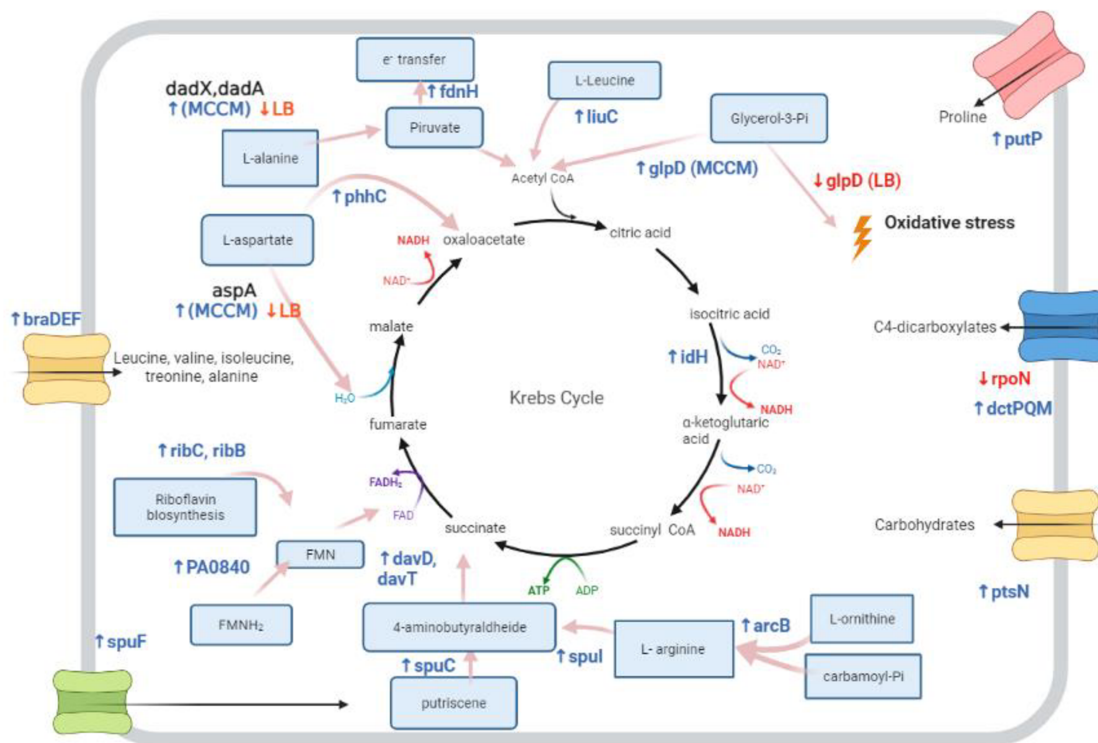


Figure 3.5- Up and downregulated *P. aeruginosa* PA01 genes observed in both LB and MCCM media at early infection stage. The genes highlighted here are known to be involved in metabolic pathways that are connected with energy production through TCA cycle or transport of intermediates. Upregulated genes are in blue and downregulated genes are in red. Only genes in bold and the *glpD* gene do not have the same response for different media: these are upregulated in MCCM and downregulated in LB.

Table 3.1- Common differentially expressed *P. aeruginosa* PA01 genes at 5 min post-infection in both LB and MCCM media. Green cells indicate upregulation, red cells indicate downregulation, and yellow cells indicates $p < 0.05$.

Gene	Annotation	COG category	Functional Category	LB t=5min		MCCM t=5min		LB t=10min		MCCM t=10min		LB t=15min		MCCM t=15min	
				Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj
PA0265	<i>davD</i>	[C] Energy production and conversion	Metabolism	0.6	4.90E-02	1.1	6.00E-03	0.67	0.02	1.29	3.95E-03	0.79	0.12	0.26	0.79
PA0840	Probable NADH:flavin oxidoreductase	[C] Energy production and conversion		2.5	1.38E-14	1.8	1.00E-03	0.64	0.35	1.34	0.18	-0.02	0.99	0.51	0.80
PA1127	Probable oxidoreductase	[C] Energy production and conversion		-1	1.00E-03	-1.6	1.00E-03	-1.41	2.12E-05	-2.11	1.88E-04	-1.63	4.88E-04	-2.06	2.44E-03
PA2624	<i>idh</i>	[C] Energy production and conversion		0.6	4.50E-02	1.6	3.40E-05	1.08	1.29E-03	1.29	5.00E-03	0.49	0.39	0.62	0.34
PA4811	<i>fdnH</i>	[C] Energy production and conversion		1.8	3.23E-12	1.5	2.00E-03	0.88	1.06E-02	-0.53	0.82	-0.09	0.95	0.23	0.93
PA0266	<i>davT</i>	[E] Amino acid transport and metabolism		0.6	4.20E-02	1.2	3.00E-03	1.28	1.64E-05	2.02	7.14E-08	1.32	3.25E-04	1.22	9.76E-03
PA0296	<i>spuI</i>	[E] Amino acid transport and metabolism		0.8	4.00E-03	1.2	1.00E-03	0.44	0.25	0.37	0.68	0.49	0.51	-0.73	0.36
PA0298	<i>spuB</i>	[E] Amino acid transport and metabolism		0.9	1.00E-03	1.2	1.00E-03	1.00	0.02	0.48	0.51	0.35	0.68	-1.31	0.01
PA0299	<i>spuC</i>	[E] Amino acid transport and metabolism		1	2.21E-05	1.3	3.00E-03	1.76	6.55E-06	1.00	0.07	0.95	0.01	-0.66	0.44
PA0302	<i>spuF</i>	[E] Amino acid transport and metabolism		0.7	4.20E-02	1.1	6.00E-03	0.35	0.59	0.57	0.37	0.11	0.94	-0.70	0.32
PA0783	Sodium proline transport	[E] Amino acid transport and metabolism		0.7	3.10E-02	1.3	2.00E-03	0.76	0.08	1.96	3.02E-05	0.66	0.26	0.81	0.30
PA0789	Probable amino acid permease	[E] Amino acid transport and metabolism		1.1	4.19E-04	1.4	1.20E-02	1.33	3.70E-03	0.38	0.80	0.34	0.71	-0.49	0.76

PA0870	<i>phhC</i>	[E] Amino acid transport and metabolism		1.2	3.35E-05	1.2	1.00E-03	0.73	4.97E-02	0.03	0.98	0.03	0.99	-0.75	0.21
PA1071	<i>braF</i>	[E] Amino acid transport and metabolism		1	1.80E-02	0.9	4.10E-02	1.41	1.00E-04	-0.67	0.53	0.94	0.37	-1.27	0.05
PA1072	<i>braE</i>	[E] Amino acid transport and metabolism		1.1	5.00E-03	1.2	3.00E-03	1.14	4.03E-03	-0.23	0.88	0.79	0.35	-1.07	0.42
PA1073	<i>braD</i>	[E] Amino acid transport and metabolism		1.2	8.00E-03	1.1	1.00E-02	1.11	0.07	-1.11	0.24	1.33	0.18	-0.31	0.86
PA1417	Probable decarboxylase	[E] Amino acid transport and metabolism		1.7	3.00E-03	2.3	4.50E-02	2.40	1.32E-05	1.17	0.68	2.29	4.70E-03	-3.54	0.40
PA5172	<i>arcB</i>	[E] Amino acid transport and metabolism		4.8	3.43E-13	1.2	5.00E-03	3.62	8.13E-19	1.27	0.02	1.32	0.07	0.80	0.38
PA2500	Probable major facilitator superfamily (MFS) transporter	[G] Carbohydrate transport and metabolism		-2	9.19E-07	-2.2	2.50E-02	-2.29	1.37E-04	-2.08	0.34	-2.52	0.00	-0.83	0.81
PA4464	<i>ptsN</i>	[G] Carbohydrate transport and metabolism		-1.2	5.00E-03	-1.1	1.70E-02	-1.41	3.44E-03	-0.92	0.32	-1.27	0.17	-1.54	0.06
PA5167	<i>dctP</i>	[G] Carbohydrate transport and metabolism		1.9	5.86E-10	1.5	1.32E-06	2.79	3.87E-16	0.11	0.94	1.37	0.01	-0.93	0.18
PA5168	<i>dctQ</i>	[G] Carbohydrate transport and metabolism		1.7	1.71E-06	1.9	1.10E-06	2.07	2.62E-07	1.27	0.05	1.04	0.32	0.08	0.98
PA5169	<i>dctM</i>	[G] Carbohydrate transport and metabolism		1.4	3.15E-04	2.1	2.34E-09	2.34	2.20E-07	1.68	3.04E-03	0.99	0.32	0.38	0.81
PA4054	<i>ribB</i>	[H] Coenzyme transport and metabolism		1.1	4.67E-04	1.1	4.10E-02	1.06	1.13E-03	1.22	0.02	-0.18	0.88	0.66	0.50
PA4055	<i>ribC</i>	[H] Coenzyme transport and metabolism		1.3	2.27E-05	1	2.80E-02	0.97	4.76E-03	1.03	0.12	0.17	0.90	0.19	0.91
PA2013	<i>liuC</i>	[I] Lipid transport and metabolism		2.2	4.00E-03	1.1	1.00E-02	1.84	0.05	0.07	0.97	0.56	0.90	-0.34	0.82
PA2550	Probable acyl-CoA dehydrogenase	[I] Lipid transport and metabolism		2	8.10E-09	1.9	9.18E-06	0.62	0.44	0.30	0.88	0.38	0.87	-0.15	0.96
PA2976	ribonuclease E	[J] Translation, ribosomal	Information storage and processing	1	1.00E-03	2	5.04E-09	0.19	0.67	1.13	0.02	-0.15	0.89	0.45	0.56

		structure and biogenesis													
PA4740	<i>prp</i>	[J] Translation, ribosomal structure and biogenesis		0.8	2.90E-02	1.4	1.22E-04	0.53	0.17	1.88	0.00	0.07	0.95	1.10	0.01
PA4936	Probable rRNA methylase (23 S)	[J] Translation, ribosomal structure and biogenesis		0.9	7.00E-03	1	2.10E-02	-0.24	0.66	0.85	0.15	-0.73	0.46	-0.62	0.49
PA1097	<i>fleQ</i>	[K] Transcription		-1.1	5.50E-06	-1.2	1.00E-03	-1.00	4.67E-04	-1.06	0.02	-0.81	0.09	-1.39	1.66E-03
PA4462	<i>rpoN</i>	[K] Transcription		-0.8	2.10E-02	-1.8	3.10E-04	-0.95	3.52E-03	-1.54	0.02	-1.07	0.01	-1.77	3.81E-03
PA4581	<i>rtcR</i>	[K] Transcription		-0.8	2.10E-02	-1.6	4.00E-03	-0.55	0.30	-1.31	0.22	-0.03	0.99	-1.36	0.33
PA4745	<i>nusA</i>	[K] Transcription		0.9	1.20E-02	2.2	1.68E-10	0.65	0.04	1.61	1.88E-04	0.49	0.45	1.68	5.65E-05
PA5255	<i>algQ</i>	[K] Transcription		-0.7	1.70E-02	-1.7	8.25E-08	-1.14	1.41E-03	-1.97	4.41E-07	-1.36	0.00	-2.10	6.25E-07
PA3159	<i>wbpA</i>	[M] Cell wall/membrane/envelope biogenesis	Cellular processes and signalling	0.8	8.00E-03	1	4.60E-02	0.27	0.62	0.06	0.97	-0.26	0.73	-0.31	0.78
PA0623	Probable bacteriophage tail tube protein	[S] Function unknown	Poorly characterized	0.9	1.40E-02	0.9	4.80E-02	2.55	2.28E-12	2.50	7.20E-10	2.03	5.84E-07	2.58	4.46E-09
PA3234	Probable sodium: solute symporter	[S] Function unknown		1.3	2.00E-03	0.9	2.50E-02	1.60	0.01	-0.41	0.66	0.35	0.85	-0.58	0.58
PA4611	Hypothetical protein	[S] Function unknown		-2.5	3.40E-02	-1.7	3.97E-04	-1.96	0.14	-1.17	0.10	-3.40	0.02	-1.28	0.12
PA4746	Hypothetical protein	[S] Function unknown		0.8	2.80E-02	2.5	5.81E-06	1.17	5.76E-04	2.16	8.62E-04	0.39	0.57	2.15	1.06E-03
PA3496	Hypothetical protein	[S] Function unknown		-0.9	6.00E-03	-2.2	2.59E-06	-0.75	0.09	-0.48	0.77	0.16	0.88	0.09	0.97
PA3233	Hypothetical protein	[T] Signal transduction mechanisms	Cellular processes and signalling	1.3	1.40E-02	1.5	2.00E-03	1.82	2.60E-05	-1.29	0.09	0.48	0.78	-1.17	0.23

3.3.4.2. Impact of phage infection on the host's two-component regulatory systems

Among the 56 PAO1 genes targeted at 5min post-infection, many are involved in two-component regulatory systems (*wspA*-chemotaxis family, **C4-dicarboxylates transport** and *RpoN*-Ntrc family, *FdnH*-NarL family, *braDE*-OmpR family, *arcB*-OmpR family), and have similar expression profiles in both media. It is worth noting that the observed genes are all upregulated, with the exception of transcriptional regulator RpoN, which is not consistent with *dctPQM* genes expression. Apart from the *wspA* gene, which encodes a probable chemotaxis transducer, the majority of these genes are involved in metabolism (*braDE*: transport of amino acids, *arcB*: L-arginine metabolism, *dctPQM* genes: transport of c4-dicarboxylates, *fdnH*: nitrate respiration, *rpoN*: transcriptional repressor *dctPQM* genes). According to KEGG pathways, all mentioned genes are also part of two-component systems, which are responsible for bacterial recognition and transduction of certain signals.

3.3.4.3. Impact of phage infection on replication-, transcription- and translation-associated genes

Expression levels of bacterial genes involved in DNA replication, transcription (transcriptional regulators, RNA repair systems, rRNA methylation) and translation (mRNA degradation and processing) represent a second category of genes influenced by phage infection. These include elongation factor G, ribonuclease E, *fleQ*, *pnp*, rRNA methylase, *rpoN*, *rtcR*, *nusA*, and *algQ* genes. These genes are differentially transcribed after phage infection in different media, leading to the same response (except elongation factor G and ribonuclease HII), yet a little more attenuated in MCCM medium.

3.3.5. Growth medium influences gene expression levels at early infection

Our initial experiments show that the growth media has only a minor influence on the phage transcriptome but greatly affect bacterial transcripts. In this section it is highlight the specific host responses during early infection that are driven by the growth medium. Table 3.2 presents PAO1 genes that were significantly, yet differentially expressed at early infection between both media.

A first example is *g/pD* gene, that is upregulated in MCCM medium and downregulated in LB medium, indicating a higher necessity of the expression of this gene in MCCM. Another bacterial gene that was upregulated in MCCM yet downregulated in LB medium after phage infection is *thiI* gene. Additionally, a clearly differentiated response between LB and MCCM during phage infection was also observed for *dadX* and *dadA*, where are upregulated in MCCM medium, but in LB medium, are downregulated.

Beyond leading to a different transcription of metabolic genes during phage infection, the growth medium also influenced genes with functions related with transcription and translation. Namely, the gene that encodes ribonuclease HII (degrades RNA from RNA-DNA hybrids) [27] is targeted after phage infection, being downregulated in LB but upregulated in MCCM medium. Similarly, elongation factor G (*fusA2*), that is responsible for the catalysation of GTP-dependent ribosomal translocation step during translation elongation, an important step during translation [28], is upregulated in MCCM but the same is not observed for LB media. Another interesting observation is the differential transcription for the transcriptional regulator *betI*. This gene is upregulated in MCCM medium and downregulated in LB medium. When transcribed, *betI* represses *betT*, that is involved in choline transport and represses *betAB* genes expression, which in turn are involved in choline oxidation to generate glycine betaine [29].

Table 3.2- Differentially expressed *P. aeruginosa* PA01 genes at 5 min post-infection that have opposite expression according to the medium. Green cells indicate upregulation, red cells indicate downregulation, yellow cells indicate $\text{padj} < 0.05$.

Gene	Annotation	COG category	Functional Category	LB t=5min		MCCM t=5min		LB t=10min		MCCM t=10min		LB t=15min		MCCM t=15min	
				Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj	Log2 Fold	padj
PA3584	<i>glpD</i>	[C] Energy production and conversion	Metabolism	-1.3	1.00E-03	2.5	1.32E-06	-1.03	2.28E-02	1.51	9.88E-02	-0.27	8.06E-01	0.41	8.81E-01
PA2204	Probable binding protein component of ABC transporter	[E] Amino acid transport and metabolism		-2	9.32E-05	2.9	3.70E-15	-1.06	9.15E-02	1.49	6.39E-02	-0.37	8.06E-01	0.78	6.26E-01
PA5304	<i>dadA</i>	[E] Amino acid transport and metabolism		-0.9	2.20E-02	2.8	7.28E-10	0.10	8.54E-01	3.82	4.45E-20	0.67	2.29E-01	2.69	1.21E-08
PA5429	<i>aspA</i>	[E] Amino acid transport and metabolism		-1.2	4.68E-06	1.9	5.00E-03	-0.67	2.88E-02	3.02	2.91E-06	-0.53	3.63E-01	2.75	5.32E-05
PA0975	Hypothetical protein	[H] Coenzyme transport and metabolism		-0.8	4.00E-03	1.1	1.60E-02	-0.43	2.36E-01	0.23	8.77E-01	-0.16	9.08E-01	0.52	6.60E-01
PA5118	<i>thiI</i>	[H] Coenzyme transport and metabolism		-0.9	1.00E-03	1.1	3.30E-02	-1.05	5.70E-05	1.32	3.06E-02	-0.88	5.46E-02	1.28	4.40E-02
PA2557	Probable AMP-binding enzyme	[I] Lipid transport and metabolism		1.2	3.50E-02	-1	1.40E-02	1.48	7.41E-03	-2.14	1.67E-04	1.27	2.83E-01	-3.29	4.52E-08
PA2071	elongation factor G	[J] Translation, ribosomal structure and biogenesis		1.1	1.00E-03	-1.2	3.00E-03	0.74	1.25E-01	-1.15	4.50E-02	0.41	7.65E-01	-1.61	3.12E-02

PA0483	Probable acetyltransferase	[K] Transcription	Information storage and processing	1.1	4.10E-02	-1.6	2.00E-03	0.37	6.07E-01	-2.20	2.25E-03	0.36	8.39E-01	-3.65	2.14E-03
PA0528	Transcriptional repressor	[K] Transcription		-1.2	6.00E-03	1.8	4.70E-02	-1.08	1.98E-02	1.48	4.27E-01	-0.74	4.47E-01	-0.45	9.50E-01
PA5374	<i>betI</i>	[K] Transcription		-1.3	1.00E-03	4.5	2.09E-16	-1.63	5.78E-05	4.55	8.04E-16	-1.34	2.25E-02	4.35	3.08E-07
PA3642	ribonuclease HII, <i>rnhB</i>	[L] Replication, recombination and repair		-0.8	3.20E-02	1	2.70E-02	-1.64	1.64E-05	0.45	6.90E-01	-1.30	1.09E-02	0.14	9.48E-01
PA5302	<i>dadX</i>	[M] Cell wall/membrane/envelope biogenesis	Cellular processes and signaling	-1	9.00E-03	2.9	4.67E-07	-0.18	7.59E-01	3.65	6.07E-16	0.51	5.36E-01	2.88	3.62E-08
PA1181	Hypothetical protein, sensor protein	[T] Signal transduction mechanisms	Cellular processes and signaling	-0.9	2.00E-03	1.1	9.00E-03	-0.71	5.00E-02	1.21	2.72E-02	0.02	9.89E-01	0.62	5.73E-01

3.4. Discussion

3.4.1. Phage transcripts are not impacted by the growth media but bacterial transcripts are markedly affected

A global tracking of bacterial and phage transcripts over time indicated that the host machinery is required to a high degree for phage reproduction as observed previously [16], and it appears that this pattern is independent of the used media. Independent of growth conditions, it is also the phage transcriptional map, which suggests a logical progression from early (host adaptation), to middle (DNA replication) and late (structural and lysis proteins) genes expression for this phage [16]. A slight delay in phage transcription was observed for the MCCM-grown infected cultures as a probable result of reduced bacterial fitness in this media, since that starvation and metabolic state have been already shown to impact phage infection parameters [30]. This phenomenon is also observed in the phage burst size, that variates according to bacterial growth rate, as demonstrated by Nabergoj and collaborators that showed that the burst size of T4 phage increases linearly with the increase of *E. coli* k-12 growth rate [31]. Nevertheless, this minor discrepancy does not impact the results obtained for phage transcriptome in both media, since that overall, phage gene expression levels do not differ between the LB and MCCM grown bacterial cultures.

Similarly, the impact of the media on bacterial transcriptome during phage infection was intended to be analysed, considering that media has a significant impact on bacterial behaviour, as mentioned in a study by Wagner and collaborators, which highlighted that the expression levels of QS genes is

influenced by growth medium [32]. Here it was founded a high number of differentially expressed genes specially with categories related with metabolism, transcription, and cell wall/membrane/envelope biogenesis. In particular, genes regulated by iron availability were induced in MCCM media comparing with LB media, indicating iron starvation in MCCM medium. This is consistent with *in vivo* host conditions, where iron availability is low due to the presence of iron binding proteins [33], and with the fact that mechanisms of iron uptake are related to *P. aeruginosa* virulence and pathogenicity in lung infections [34]. Consistently, genes involved in bacterial pathogenicity and virulence were also induced under MCCM relative to LB medium, including genes involved in secretion systems (type I, II, III and IV), two-component system, biofilm formation, QS, multidrug resistance and virulence factors. Thus, all together these difference between MCCM and LB media proves that the composition of the media drastically influences the *P. aeruginosa* pathogenicity and virulence in addition to the bacterial metabolism. Within the focus of this research, these differences among the media serve as a control, enabling teasing out the phage-infection induced transcriptional responses between both cultures.

3.4.2. Elucidating the bacterial response to phage infection

When the total transcriptome is examined during infection, the major bacterial responses to phage infection occur during the early infection period, as observed previously [5]. Therefore, this early phase is critical to reveal potential bacterial defence mechanisms, as well as processes triggered by phage for its infection cycle, since that there is a transcriptional convergence observed for the middle and late samples for both growth media. This indicates that during the course of infection, the impact of the growth medium is nullified, and that the transcriptional differences between samples are impacted by the high expression of phage genes and by bacterial genes targeted by phage. Thus, during middle and late infection, bacterial responses that are associated with growth medium conditions decrease relative to responses triggered by phage infection.

3.4.2.1. Phage infection impact in energy metabolism regardless of bacterial growth conditions

Considering the importance of early infection stage, differentially expressed genes in both growth conditions were tracked, indicating a strong manipulation of metabolic pathways, particularly related with energy production through TCA cycle. An example, is the induction of pathways related with C4-dicarboxylates acquisition, being these known to be the *P. aeruginosa* preferential carbon and energy source [35,36]. This suggests that during early infection, the transport of these substrates is crucial to obtain high levels of energy. This hijacking of molecular resources is consistent with the coliphage T4

infection process, and it is associated with energy requirement for phage particle formation [37]. The changes in metabolism observed in LUZ19-infected *P. aeruginosa* cultures at early stage are driven by phage at the very beginning of infection to assure the progression of infection.

Another example is the induction of riboflavin and its biological active forms, which are widely used as cofactors by bacterial enzymes during redox reactions [38], but when its biosynthesis is inhibited, it leads to nutrient deficiency and oxidative stress [39]. The differentially expressed genes associated with vitamins and cofactors biosynthesis suggests that phages require their presence during infection. Correspondingly, an additional clue towards energy metabolism is provided by the upregulation *fdnH* gene, that is involved in the conduction of electrons from formate to nitrate. This enzyme has particular relevance during nitrate respiration that often occurs under anaerobic conditions, being the gene expression induced in the presence of nitrate [37]. In this dataset, the expression of the β -subunit of this protein is upregulated in early infection, compared with uninfected cultures and regardless of the media. This indicates that under phage infection, the expression of formate dehydrogenase-N might be required since this enzyme is responsible for proton motive force generation that ultimately results in energy production.

Metabolic pathways that are also triggered after phage infection involve the conversion of putrescine to produce succinate, a TCA intermediate, and ultimately leads to the production of energy. However, related with this pathway, the transport of putrescine and spermidine is also induced, which hints at a structural relevance for spermidine transport, beyond the purpose of energy acquisition [40]. Indeed, it was previously suggested that spermidine compacts and stabilizes viral DNA in the capsid [41,42], and it is essential for N4-like *Pseudomonas* phages infection cycle [39]. This is a good example of multipurpose genes that are probably activated by phage infection to participate, in this specific case, in two mechanisms such as energy generation and phage DNA stabilization.

3.4.2.2. Impact of phage infection on the host's two-component regulatory systems

The two-component regulatory systems consist of mechanisms that enable *P. aeruginosa* PAO1 to sense and respond to environmental stimuli and potentially to phage infection. The results demonstrated several genes related with two-component system being altered. Among them was *rpoN* that is reported to positively regulate *dctPQM* genes [36], although in this data, expression of this transcriptional regulator is not consistent with *dctPQM* genes expression. Still, C4-dicarboxylates abundance stimulates the expression of its own transporters, such as *dctPQM* gene products. Another example is gene *fdnH*, that is triggered upon nitrate presence that works as a signal for promotion of nitrogen metabolism.

Other two-component system involved gene that was impacted by phage infection according to the obtained results were genes involved in the signal transduction of anaerobic respiration inhibition by quinone electron carriers (*arcB* gene). Other signal mechanisms targeted during early infection were: the signal mechanism that promotes bacitracin efflux when it is abundant (*braDE* genes); and signal obtained from surface cell contact that induces extracellular polysaccharide production (*wspA*). Also, RpoN participates in several signal-response mechanisms that results in a myriad of activities: the induction of amino acids uptake and metabolism; attaching and effacing (AE) lesions; *pilA* expression; regulation of biofilm formation, motility and virulence; flagellar assembly. In future, it would be of interest to understand whether the upregulation of two-component regulatory system genes is a bacterial answer to phage infection or if these genes are induced by phage for successful infection.

Presumably, the higher expression levels of genes involved in two-component regulatory systems represent a bacterial response to phage infection, as suggested in a previous work, in which the upregulation of *pqsABCDE* operon was proposed to function as a signal to shutdown bacterial metabolism of non-infected cells [7]. While expression of *pqs* operon was higher during late infection in LB, this difference wasn't significant compared to uninfected cultures. By contrast, the operon was significantly downregulated in MCCM, highlighting the importance of growth conditions during infection assays that also contribute to changes in gene expression.

3.4.2.3. Impact of phage infection on replication-, transcription- and translation-associated genes

Several changes were observed concerning the transcription process at early infection, including changes in the expression of genes involved in mRNA degradation (*pnp* gene-polyribonucleotide nucleotidyltransferase, and ribonuclease E) [43,44], indicating that, at early infection, mRNA turnover is tightly controlled by the phage. Another major change was related with transcription termination/antitermination protein (*nusA*), indicating the necessity of a strict regulation of transcriptional mechanisms during phage infection. The upregulation of this gene might be part of an underlying mechanism that phage deploys to tightly control host machinery. It would be interesting to investigate this mechanism further in future.

In addition, a tight regulation of RNA turnover early in infection is also confirmed by the differential expression of RNA repair system regulators *rpoN* (RNA polymerase sigma-54 factor) and *rtcR* (transcriptional regulator of *rctAB* genes) which are downregulated independent of the growth medium. Besides *rpoN* transcriptional regulator expression was not consistent with *dctPQM* genes, its transcription was consistent with *rctAB* operon system expression, indicating that the upregulation of this

transcriptional regulator has as purpose the influence of RNA turnover mechanisms. Additionally, RtcR is known to work through RpoN to positively activate the expression of *rctAB* operon that encodes for two constituents of tRNA repair system [45]. The downregulation of these two regulators indicates that the RNA repair system is repressed, which would be distinct from the *P. aeruginosa* strain PAK infected by phage PAK_P3, where the expression of *rctAB* was significantly upregulated [5]. This indicates that a broad transcriptional regulator, such as RpoN, functions in distinct ways in bacterial responses and other mechanisms are probably associated to ensure expression regulation.

Two additional transcriptional regulators are significantly targeted at this early phase: transcriptional regulator *fleQ* and *betI*. FleQ, together with FleR, is involved in a regulatory cascade that controls motility and adhesion in *P. aeruginosa* [46]. Considering the crucial role of *fleQ* in the control of motility and adhesion, its downregulation can lead to a non-control of this important bacterial aspect, which might impact bacterial infection establishment. Similarly, the downregulation of global transcriptional *P. aeruginosa* regulator and repressor of *lasR* and *rhl*, the *algQ* gene, might suggest that bacterial control is no longer available, leading to a fail in the regulation of QS systems [47]. With this data it was not possible to determine if the shutdown of bacterial functions such as QS, motility and adhesion would be beneficial for phage infection, being this a probable mechanism of phage to manipulate its host.

3.4.3. Phage targets differently specific metabolisms to assure successful infection

Under different media, at early infection, phage manipulates glycerol metabolism. In MCCM medium *gpd* is induced, but not in LB. This gene encodes a glycerol-3-phosphate dehydrogenase (G3P), which acts as a key player in energy metabolism (involved in glycerol metabolism). It is known that the complete abolishment of this gene leads to alterations in amino acids metabolism and in the production of TCA intermediates, ending in a general stress response [48]. The tight control of this gene expression seems to be imperative for proper metabolism functionalization. As such, it appears to be an essential gene to target to achieve ideal infection conditions. A similar behaviour is observed for gene *thiI* that codes for a protein involved in the biosynthesis of thiazole (precursor of thiamine -vitamin 1). In bacteria, thiamine is an important cofactor with a fundamental role in energy metabolism [48]. The induction of this gene exclusively in MCCM media might be associated with the low concentration of this vitamin 1 precursor in this medium, which in the infection context might be highly required.

Alanine metabolism-associated genes (*dadAX* genes) seem to be important under MCCM medium presence. The presence of L-alanine (prevenient from intrinsic metabolism) is reported to be the

main inducer of *dadAX* genes [49], as well as nitrogen starvation. The obtained results demonstrate that possibly, the MCCM medium represents an energy starvation condition for PAO1, contrary to LB, which would activate these genes to produce nitrate and in pyruvate fermentation. Indeed, it was previously reported that nitrate can serve as alternative of electrons acceptor under anaerobic conditions in PAO1, and that pyruvate fermentation plays an important role as a general endogenous survival metabolism in response to energy starvation [50]. As such, the use of MCCM medium might better mimic anaerobic conditions, typical of biofilm and cystic fibrosis infection conditions, as reflected by the shift in amino acids metabolism.

Finally, in MCCM medium, an induction of *betI* reveals a potential increase in glycine betaine production. This product is reported to function as an osmo-protector, promoting *P. aeruginosa* virulence and survival in mouse lung [29]. Inhibition of choline-betaine pathway is highly toxic to *P. aeruginosa* but upon phage infection in MCCM medium, the upregulation of *betI* gene leads to the repression of *betAB* genes, which was also observed in PaP1-infected *P. aeruginosa* [51].

Overall, this work was able to show differences in bacterial gene expression patterns following phage infection in LB compared to MCCM, and it is tempting to speculate that these changes are triggered by the phage, although a response of the bacterium in this medium, in the presence of phage remains equally plausible. Regardless, these data suggest optimal conditions for phage propagation are attained, in view of the expression profile convergence later in infection.

3.5. Conclusions

RNA-seq of LUZ19 - infected *P. aeruginosa* PAO1 cultures demonstrates that major transcriptional changes occur at the early infection stage, during bacterial takeover. This early expression stage is marked by specific changes in expression levels, targeting energy metabolism, translation and transcription. A medium-specific response to phage infection was also observed, targeting a handful of regulatory genes, that share a common response in terms of adapting to the limitations imposed MCCM medium compared to lysogeny broth. It is interesting that this response visualized at early infection doesn't influence LUZ19 infection that proceeds largely uninterrupted and efficiently, despite these limitations.

From this chapter, it is tempting to speculate that this small, yet densely coded phage has the genomic versatility to readily adapt the host machinery towards its needs. This would cast a different light on the types of analyses needed to understand some of the 'viral dark matter' genes, and their role under non-standard growth conditions.

From a phage therapy perspective, a first step towards assessing the efficiency and impact of this phage in MCCM medium was taken, paving the way to uncover the phage infection process in conditions comprising the three partners involved: phage-bacteria-human cell. Within this context, the versatility shown by LUZ19 appear promising, as this phage is well adapted to the imposed different environmental conditions.

3.6. References

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3.7. Supplementary material

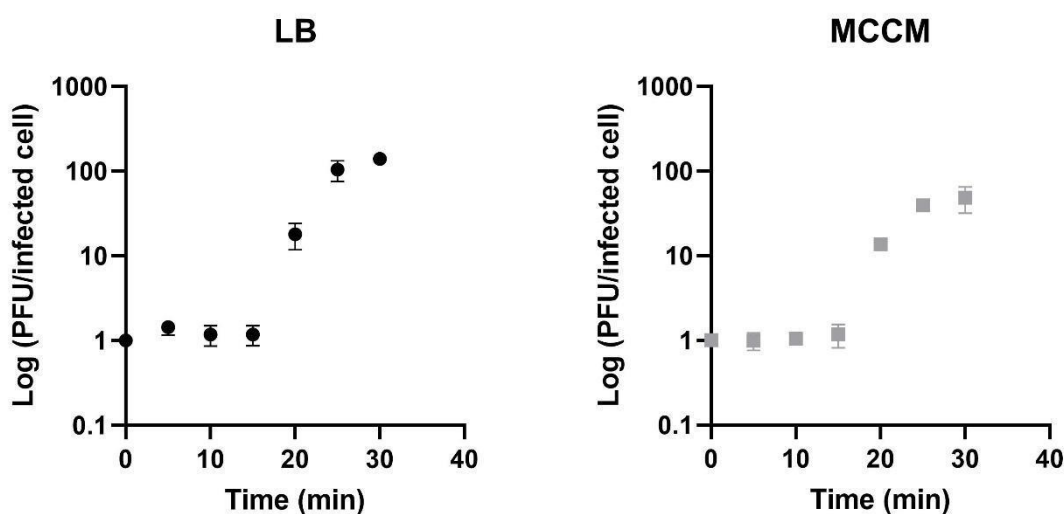


Figure S3.1- OSGC of LUZ19 phage on LB or MCCM medium. Bacterial cultures grown until OD=0.3 were infected with phage suspensions at a MOI=0.01. Bars represent standard deviations.

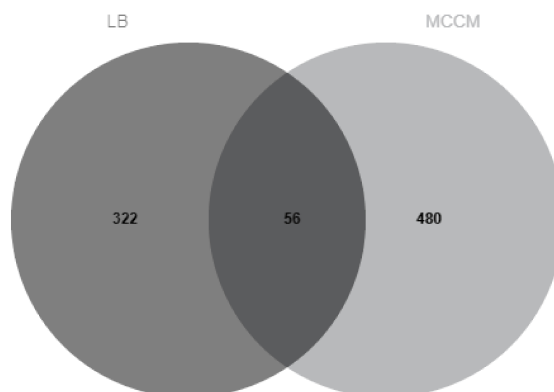


Figure S3.2- *P. aeruginosa* PAO1 genes differentially expressed at early infection (t=5min).

Venn diagram shows the interception of LB and MCCM conditions, where 56 genes are commonly significantly regulated.

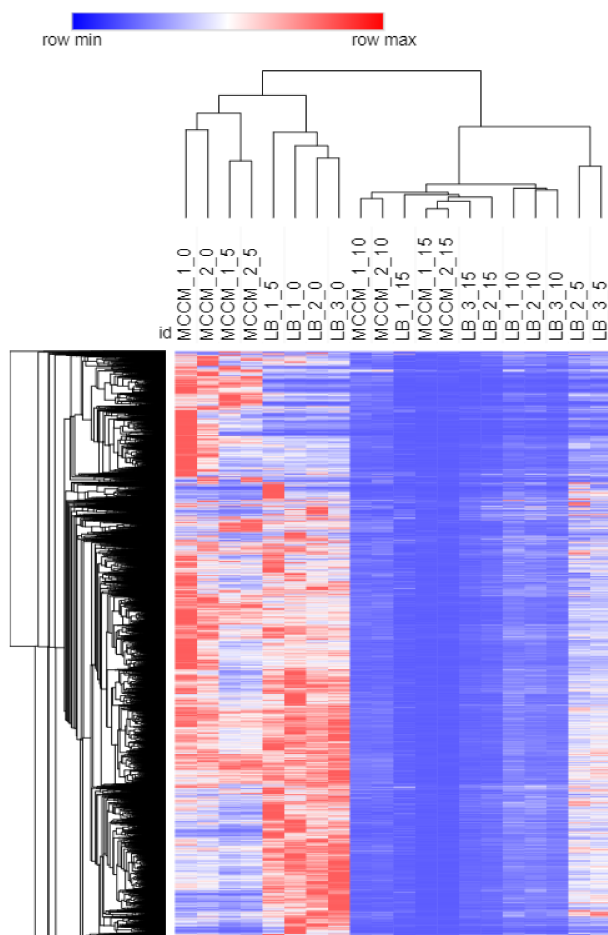


Figure S3.3- Heat map of *P. aeruginosa* PAO1 genes expression in different time points of synchronized assays performed in LB and MCCM medium. Expression values were adjusted by z-score and Hierarchical clustering was performed using Euclidian distance.

Table S3.1- Upregulated genes in MCCM medium relative to LB medium. These genes are involved in iron acquisition and their expression at early infection in both medium is represented.

Iron upregulated genes in MCCM VS LB (t=0min)				LB (5 vs 0min)		MCCM (5 vs 0min)	
Gene	Log2Fold	padj	Name	Log2Fold	padj	Log2Fold	padj
Ferric citrate sensing: signal transduction mechanism							
PA1364	2.28	6.47E-05	Probable transmembrane sensor: periplasmic ferric-dicitrate binding protein ferR. regulates iron transport through sigma-19	0.13	9.63E-01	-3.46	2.59E-06
PA3899	2.72	7.95E-06	FecI	-0.84	7.58E-01	-2.38	5.46E-03
PA3900	2.42	1.67E-04	Periplasmic ferric-dicitrate binding protein FerR, regulates iron transport through sigma-19	-1.35	4.52E-01	-3.24	9.01E-05
PA5217	3.12	3.68E-15	Probable binding protein component of ABC iron transporter	-0.53	5.11E-01	-4.25	1.19E-24
PA4895	5.14	1.74E-23	Probable transmembrane sensor	-0.45	8.78E-01	-2.50	8.74E-09
Transcriptional regulators							
PA1300	4.05	1.26E-30	ECF sigma factor: DNA directed RNA polymerase specialized sigma subunit, sigma24 family	0.09	9.72E-01	-3.89	2.01E-24
PA1363	2.21	2.24E-07	ECF sigma factor: DNA-directed RNA polymerase specialized sigma subunit, sigma24 family	0.41	7.12E-01	-3.54	1.04E-09
PA4896	4.49	7.29E-04	Probable sigma-70 factor, ECF subfamily	-0.89	8.29E-01	-3.47	2.60E-17
Iron harbouring proteins							
PA3530	2.74	3.26E-11	Bacterioferritin-associated ferredoxin, Bfd	0.19	9.13E-01	-3.96	1.34E-12
PA4615	2.54	4.84E-19	FprB	-0.03	9.87E-01	-0.05	9.73E-01
PA2475	2.23	3.75E-05	Probable cytochrome p450	0.59	7.48E-01	-0.21	8.84E-01

Siderophores transport							
PA2466	2.59	1.92E-11	Ferrioxamine receptor, FoxA: siderophore transport	-0.26	8.79E-01	-0.65	3.85E-01
PA2467	3.67	1.13E-21	Anti-sigma factor Foxr	0.12	9.63E-01	-4.24	9.19E-15
PA2468	4.50	2.30E-36	ECF sigma factor FoxI	0.09	9.67E-01	-4.18	8.80E-23
PA4156	3.23	1.67E-10	FvbA, siderophore transport	1.10	2.62E-01	-0.24	8.63E-01
PA4675	2.88	1.03E-12	ChtA: siderophore transport	-0.63	2.84E-01	-0.53	4.61E-01
PA1365	2.40	3.91E-13	Probable siderophore receptor: outer membrane receptor for ferrienterochelin and colicins	-0.10	9.63E-01	0.29	7.67E-01
PA1910	2.42	8.76E-09	Ferric-mycobactin receptor, FemA	-0.32	8.78E-01	-0.57	4.81E-01
PA1911	4.77	1.44E-16	Sigma factor regulator, FemR	0.43	9.06E-01	-3.09	2.23E-05
PA1912	5.19	4.27E-23	ECF sigma factor, FemI	1.63	1.48E-01	-2.74	6.31E-07
PA2033	7.52	1.02E-90	Hypothetical protein: fad-binding domain, ferredoxin reductase-type	0.39	8.32E-01	-2.08	1.16E-06
PA0931	3.67	3.11E-32	Ferric enterobactin receptor PirA: outer membrane receptor for ferrienterochelin and colicins	-0.44	6.81E-01	-1.08	1.23E-02
PA4160	3.36	9.09E-05	Ferric enterobactin transport protein FepD	1.27	6.34E-01	-2.65	3.48E-02
Ferripyoverdine							
PA2398	5.21	3.53E-67	Ferripyoverdine receptor, FpvA	-0.54	2.62E-01	0.91	1.01E-01
PA2409	2.61	3.12E-13	FpvE	-0.20	8.90E-01	0.87	5.67E-02

PA2403	4.04	7.03E-29	FpvG: pyoverdine biosynthetic process	-0.70	4.57E-01	0.30	6.66E-01
PA4168	2.63	4.44E-11	Second ferric pyoverdine receptor FpvB	0.15	9.06E-01	-0.34	7.18E-01
Phenazine biosynthesis							
PA1902	4.05	2.23E-03	Phenazine biosynthesis protein Phzd, PhzD2	0.12	9.89E-01	0.34	9.68E-01
PA1905	2.15	7.96E-03	Probable pyridoxamine 5'-phosphate oxidase, PhzG2	1.20	3.82E-01	1.52	2.65E-01
PA4210	6.43	8.41E-03	Probable phenazine biosynthesis protein, Phza1	2.19	3.04E-01	-0.40	9.77E-01
PA4211	5.52	4.13E-03	Probable phenazine biosynthesis protein, PhzB1	0.88	6.77E-01	-0.24	9.84E-01
PA4213	3.31	1.08E-02	Phenazine biosynthesis protein PhzD, PhzD1	-0.12	9.88E-01	1.36	8.37E-01
PA4215	2.11	2.42E-02	Probable phenazine biosynthesis protein, Phzf1	-0.01	9.98E-01	0.53	8.39E-01
PA4216	2.12	8.68E-03	Probable pyridoxamine 5'-phosphate oxidase, Phzg1	0.07	9.87E-01	1.21	4.21E-01
PA4217	3.81	2.20E-17	Flavin-containing monooxygenase, PhzS	0.57	5.39E-01	0.98	2.59E-01
Iron obtaining by transport and degradation of heme							
PA0122	2.69	2.62E-02	RahU: haemolysis by symbiont of host erythrocytes	0.07	9.63E-01	-0.40	9.49E-01
PA0672	5.72	2.38E-54	Heme oxygenase, HemO	-0.54	7.78E-01	-0.93	2.45E-02
PA0471	3.68	5.68E-22	FiuR: periplasmic ferric-dicitrate binding protein FerR, regulates iron transport through sigma-19	-0.81	6.45E-01	-3.80	4.40E-14
PA0472	4.40	1.76E-25	FiuL: DNA-directed RNA polymerase specialized sigma subunit, sigma24 family	-0.10	9.75E-01	-4.31	7.87E-19

PA1302	2.24	2.44E-09	Probable heme utilization protein precursor: outer membrane receptor proteins, mostly Fe transport	-0.44	6.38E-01	0.21	8.23E-01
Heme uptake by Has system							
PA3405	6.24	1.85E-07	Metalloprotease secretion protein, HasE	0.14	9.86E-01	1.23	6.59E-01
PA3406	4.63	1.84E-13	Transport protein HasD	0.18	9.67E-01	1.13	2.47E-01
PA3407	5.98	1.78E-48	Heme acquisition protein HasAP	0.04	9.87E-01	0.74	7.68E-01
PA3408	4.71	1.14E-35	Heme uptake outer membrane receptor HasR precursor	-0.42	7.61E-01	-1.77	8.43E-03
PA3409	6.02	1.94E-43	HasS	-0.41	8.96E-01	-3.34	1.50E-15
PA3410	5.15	3.27E-38	HasI	0.13	9.54E-01	-3.87	4.45E-24
Heme uptake by Phu system							
PA4705	2.54	3.35E-19	PhuW	-0.45	5.09E-01	0.47	4.31E-01
PA4706	3.49	7.89E-30	PhuV	0.02	9.93E-01	-0.01	9.96E-01
PA4707	3.39	1.08E-35	PhuU	-0.41	6.70E-01	-0.81	7.79E-02
PA4708	5.39	1.53E-53	Heme-transport protein, PhuT	-0.52	6.99E-01	-2.36	2.41E-10
PA4709	5.90	1.16E-65	Putative heme degradation protein	-0.02	9.93E-01	-3.38	1.16E-24
PA4710	4.84	7.25E-44	Heme/haemoglobin uptake outer membrane receptor PhuR precursor	0.54	4.94E-01	-3.23	1.71E-14
Pyochelin biosynthesis genes							
PA4221	4.87	2.61E-47	Fe(III)-pyochelin outer membrane receptor precursor (FptA), outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid	-0.68	5.02E-01	0.03	9.80E-01

PA4224	4.22	1.24E-15	Pyochelin biosynthetic protein PchG	-0.09	9.85E-01	0.78	1.60E-01
PA4225	3.93	6.86E-43	Pyochelin synthetase, PchF	-0.09	9.49E-01	0.91	3.03E-02
PA4228	4.67	1.24E-33	Pyochelin biosynthesis protein PchD	-0.80	7.48E-01	1.24	2.61E-02
PA4229	4.17	1.96E-09	Pyochelin biosynthetic protein PchC	-0.15	9.86E-01	1.10	1.59E-01
Pyoverdine biosynthesis							
PA2385	7.67	3.25E-116	PvdQ	-0.30	9.13E-01	1.14	8.79E-04
PA2386	8.52	7.18E-123	PvdA, lornithine n5-oxygenase	-0.23	9.15E-01	0.55	3.22E-01
PA2389	3.95	3.04E-25	PvdR	-0.41	7.48E-01	0.83	5.36E-02
PA2390	3.95	9.09E-37	PvdT, pyoverdine biosynthetic process	-0.73	3.15E-01	0.58	1.98E-01
PA2391	4.24	2.65E-42	Probable outer membrane protein precursor, OpmQ: pyoverdine biosynthetic process	-0.59	6.23E-01	0.60	2.23E-01
PA2392	8.58	7.12E-173	PvdP	-0.27	8.92E-01	0.28	6.48E-01
PA2393	6.00	9.19E-78	Putative dipeptidase: pyoverdine biosynthetic process, dipeptidase activity	0.68	5.75E-01	0.96	1.39E-02
PA2394	6.12	9.67E-70	PvdN	-0.78	6.99E-01	1.19	3.05E-03
PA2395	8.17	1.43E-61	PvdO	1.02	6.99E-01	0.42	5.80E-01
PA2396	8.10	6.06E-132	PvdF, pyoverdine synthetase f	0.04	9.88E-01	-0.10	9.10E-01
PA2397	4.92	1.68E-35	PvdE, pyoverdine biosynthesis protein	0.11	9.61E-01	1.54	4.06E-04

PA2399	8.03	4.72E-237	PvdD, pyoverdine synthetase d	-0.07	9.60E-01	0.23	7.36E-01
PA2400	7.46	1.05E-170	PvdJ, pyoverdine biosynthetic process	-0.35	6.57E-01	0.52	2.46E-01
PA2424	8.29	1.40E-211	PvdI	-0.22	8.58E-01	0.50	3.08E-01
PA2425	8.53	5.51E-74	PvdG	-0.82	8.46E-01	-0.72	9.94E-02
PA2426	7.85	1.96E-09	PvdS, sigma factor	0.70	8.41E-01	0.49	3.98E-01
PA2413	10.46	5.29E-159	PvdH, l-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase	0.25	9.34E-01	-0.15	8.37E-01

Table S3.2- Upregulated genes in MCCM medium comparatively to LB medium. These genes have various functions related with bacterial pathogenicity and virulence.

Interesting upregulated genes in MCCM vs LB at 0 min				LB (5 vs 0 min)		MCCM (5 vs 0 min)	
Gene	Log2Fold	padj	Name	Log2Fold	padj	Log2Fold	padj
T3SS system							
PA1706	2.35	2.28E-06	Type III secretion protein, PcrV	-0.76	3.08E-01	-0.44	7.59E-01
PA1708	4.21	2.83E-06	Translocator protein PopB: Type III Secretion pathway	-0.12	9.47E-01	-0.31	9.50E-01
PA1709	3.61	0.002	Translocator protein PopD: Type III Secretion	0.13	9.68E-01	-0.29	9.54E-01
PA1718	3.21	9.58E-16	Type III export protein, PscE	-0.63	6.77E-01	0.07	9.72E-01
PA1720	2.02	0.0003	Type III export protein, PscG	-0.35	7.66E-01	-0.24	9.00E-01
PA1721	2.90	1.94E-10	Type III export protein, PscH	-0.20	9.15E-01	0.41	7.55E-01

PA1722	2.33	1.25E-07	Type III export protein, PscI	-0.35	7.56E-01	0.36	8.03E-01
PA1725	2.71	2.35E-09	Type III export protein Psc: Flagellar biosynthesis/Type III secretory pathway protein FliH	-0.35	7.03E-01	-0.19	9.03E-01
PA0044	2.49	0.01	Exoenzyme T, ExoT	-0.05	9.77E-01	-0.44	9.21E-01
PA1714	2.28	8.15E-07	ExsD	-0.41	4.51E-01	0.33	6.60E-01
PA2191	2.33	2.33E-06	Adenylate cyclase, ExoY	-0.71	2.53E-01	-1.53	5.09E-01
PA3841	3.99	1.72E-06	Exoenzyme S	0.03	9.89E-01	-1.17	7.03E-01
PA1713	2.28	1.36E-18	Transcriptional regulator ExsA: AraC-Type DNA-binding domain and AraC-containing proteins	-0.44	4.58E-01	0.04	9.83E-01
PA1984	3.71	3.96E-19	NAD ⁺ dependent aldehyde dehydrogenase, ExaC	-0.56	7.95E-01	1.74	4.51E-04
Other secretion system							
PA0423	4.01	1.83E-51	PasP. Polyisoprenoid-binding periplasmic protein YceI, Secreted Factors (toxins, enzymes, alginate)	0.69	2.95E-02	0.16	8.63E-01
PA1249	3.22	9.05E-15	Alkaline metalloproteinase precursor, AprA: Apr Type I secretion system	0.05	9.81E-01	-0.08	9.62E-01
PA2862	3.63	2.19E-09	Lactonizing lipase precursor, LipA: metabolism and protein secretion by the Type II secretion system	1.73	1.27E-01	-0.19	9.02E-01
Type IV secretion system							
PA2360	2.98	5.26E-11	HsiA3: Predicted component of the Type VI protein secretion system	0.41	8.42E-01	-0.22	8.73E-01
PA2361	2.91	7.75E-16	IcmF3	0.53	6.40E-01	-0.24	7.95E-01
PA2362	3.33	2.84E-06	DotU3: Bacterial secretion system	-0.07	9.90E-01	-0.23	8.97E-01
PA2363	3.35	9.93E-15	HsiJ3	0.33	8.27E-01	-0.39	7.16E-01

PA2364	2.93	1.27E-14	Lip3	-0.21	9.18E-01	-0.27	7.77E-01
PA2365	2.74	6.62E-05	HsiB3	-0.29	9.37E-01	-0.59	6.67E-01
PA2366	3.17	8.55E-14	HsiC3: Predicted component of the Type VI protein secretion system	0.24	9.05E-01	0.02	9.90E-01
PA2367	2.56	5.58E-09	Type VI protein secretion system component Hcp (secreted cytotoxin), Hcp3	0.07	9.87E-01	-0.56	5.78E-01
PA2368	4.50	0.01	HsiF3	1.46	8.49E-01	-1.31	6.33E-01
PA2369	3.35	1.04E-10	HsiG3	0.27	9.33E-01	0.26	8.28E-01
PA2370	2.66	0.02	HsiH3	0.89	8.21E-01	0.65	7.73E-01
PA2371	3.66	1.00E-13	ClpV3, Type IV secretion system	-0.76	6.63E-01	0.05	9.71E-01
PA2373	2.23	8.95E-07	VgrG3. Implicated in Type VI secretion and phage assembly	0.11	9.63E-01	0.69	3.11E-01
PA2374	2.70	0.0005	TseF	-1.06	7.74E-01	-0.05	9.85E-01
Two-component system							
PA0176	2.46	4.03E-14	Aerotaxis transducer Aer2: Methyl-accepting chemotaxis protein	-0.09	9.67E-01	0.31	6.92E-01
PA0177	2.30	9.51E-08	Probable purine-binding chemotaxis protein: Chemotaxis signal transduction protein	0.05	9.87E-01	0.08	9.57E-01
PA0178	2.22	2.81E-05	Probable two-component sensor	0.12	9.63E-01	0.10	9.46E-01
PA0179	2.72	1.19E-07	Probable two-component response regulator	0.41	8.45E-01	0.19	8.96E-01
PA0180	2.20	6.30E-11	Chemotactic transducer for trichloroethylene [positive chemotaxis], CttP	-0.25	8.34E-01	-1.04	6.23E-02

PA0929	2.36	1.30E-08	Two-component response regulator: DNA-binding response regulator, OmpR family, contains REC and winged-helix (wHTH) domain	-0.20	8.81E-01	-3.95	1.81E-20
PA0930	2.73	1.06E-15	Two-component sensor: Signal transduction histidine kinase, HAMP domain	-0.69	3.53E-01	-2.55	8.29E-12
PA1342	3.33	3.57E-09	AatJ: ABC-Type amino acid transport/signal transduction system, periplasmic component/domain	1.06	2.60E-03	0.75	8.29E-01
PA0676	2.04	0.002	Sigma factor regulator, VreR. It is involved in signal transduction controlling virulence functions	-0.55	8.03E-01	-1.07	3.21E-01
PA2383	2.22	2.49218E-08	DNA-binding transcriptional regulator, LysR family	-0.60	6.77E-01	0.24	8.11E-01
PA2571	2.26	1.58E-11	Probable two-component sensor: Signal transduction histidine kinase regulating C4-dicarboxylate transport system	-0.35	7.58E-01	-0.69	2.33E-01
PA2572	2.45	0.0001	Probable two-component response regulator	0.64	7.30E-01	0.28	7.87E-01
PA2573	2.49	8.15E-18	Probable chemotaxis transducer	0.26	8.04E-01	-0.31	6.81E-01
PA2686	3.35	6.22E-22	Two-component response regulator, PfeR	-0.21	9.05E-01	-4.25	1.91E-13
PA2687	2.74	1.86E-15	Two-component sensor, PfeS	-1.10	1.07E-01	-1.94	2.82E-05
PA4290	2.74	2.62E-10	Probable chemotaxis transducer	2.31	1.07E-09	-0.76	3.18E-01
PA4296	2.80	7.54E-07	Two-component response regulator, PprB	0.26	9.13E-01	0.27	7.83E-01
PA4520	2.26	4.25E-19	Probable chemotaxis transducer	0.09	9.42E-01	-0.99	2.06E-02
PA1992	2.42	5.22E-10	ErcS: signal transduction	0.91	1.62E-01	-0.14	9.24E-01
Regulators							
PA3622	3.55	7.73E-30	Sigma factor, RpoS	0.10	9.34E-01	-1.54	7.95E-06

PA0985	3.71	4.29E-18	Pyocin S5	0.56	6.85E-01	0.28	7.47E-01
Virulence factor							
PA1041	5.72	1.61E-75	Probable outer membrane protein precursor: Outer membrane protein OmpA and related peptidoglycan-associated (lipo)proteins	0.34	8.21E-01	-1.03	6.29E-03
Biofilm involved							
PA1955	2.47	0.02	FapB	1.13	7.15E-01	-1.55	4.33E-01
Quorum sensing							
PA0602	3.06	4.10E-21	Probable binding protein component of ABC transporter: quorum sensing	1.26	1.18E-04	-0.54	3.93E-01
PA0997	2.56	8.70E-23	3-oxoacyl-[acyl-carrier-protein] synthase III, PqsB	0.81	3.36E-02	-0.82	5.92E-02
PA0998	2.28	1.07E-22	3-oxoacyl-[acyl-carrier-protein] synthase III, PqsC	0.66	5.28E-02	-0.42	4.93E-01
PA1000	2.47	2.04E-16	Quinolone signal response protein, PqsE	0.57	2.26E-01	-0.29	7.36E-01
PA1001	2.37	3.86E-19	Anthranilate synthase component I, PhnA	0.65	9.13E-02	-0.24	7.31E-01
PA1002	2.22	6.95E-12	Anthranilate synthase component II, PhnB: Anthranilate/para-aminobenzoate synthase component II	0.44	4.60E-01	0.00	9.98E-01
PA3478	3.07	2.64E-08	Rhamnosyl transferase chain B: in pathways like quorum sensing and biofilm	0.20	9.05E-01	-0.84	4.48E-01
Multidrug resistance							
PA0424	2.02	1.90E-10	Multidrug resistance operon repressor MexR:DNA-binding transcriptional regulator, MarR family	-0.18	8.84E-01	0.23	7.88E-01
PA0958	2.28	4.24E-10	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	0.75	4.20E-03	0.42	6.67E-01
PA1908	4.12	6.09E-05	Probable major facilitator superfamily (MFS) transporter: Tetracycline resistance protein signature	0.45	9.55E-01	0.54	7.14E-01

PA5542	3.64	3.66E-23	<i>Pseudomonas</i> imipenem beta-lactamase PIB-1: response to antibiotic	1.05	7.27E-03	0.15	8.70E-01
PA4218	4.48	0.0004	AmpP	-1.09	5.78E-01	1.13	1.77E-03
PA4219	5.47	1.07E-31	Uncharacterized iron-regulated membrane protein, AmpO	-0.08	9.87E-01	1.39	6.88E-04
PA3038	2.87	5.28E-14	OpdQ	1.24	4.97E-03	0.28	7.55E-01
PA3186	4.56	1.94E-28	Glucose/carbohydrate outer membrane porin OprB precursor	0.62	1.84E-01	0.68	5.46E-01

CHAPTER IV: IMPACT OF PHAGE PREDATION ON *P. AERUGINOSA* ADHERED TO HUMAN AIRWAY EPITHELIUM- ASSESSING THE MAJOR TRANSCRIPTOMIC CHANGES IN METABOLISM AND VIRULENCE-ASSOCIATED GENES

This chapter is based on following scientific article:

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Impact of phage predation on *P. aeruginosa* adhered to human airway epithelium: major transcriptomic changes in metabolism and virulence-associated genes. Under review in RNA Biology.

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Abstract

Phage therapy is a promising adjunct therapeutic approach against bacterial multidrug-resistant infections, including *Pseudomonas aeruginosa*-derived infections. Nevertheless, the current knowledge about the phage-bacteria interaction within a human environment remains limited. In this chapter, a transcriptome analysis of phage-infected *P. aeruginosa* adhered to a human epithelium (Nuli-1 ATCC® CRL-4011™) was done. To this end, RNA-seq was performed on a complex mixture comprising phage–bacteria–human cells at early, middle, and late infection and compared it to uninfected adhered bacteria.

Overall, it was demonstrated that phage genome transcription remains largely unaltered by bacterial growth and phage employ a core strategy of predation through upregulation of prophage-associated genes, a shutdown of bacterial surface receptors, and motility inhibition. In addition, specific responses were captured under lung-simulating conditions, with the expression of genes related to spermidine synthesis, sulphate acquisition, biofilm formation (both alginate and polysaccharide syntheses), LPS modification, pyochelin expression, and downregulation of virulence regulators. These responses should be carefully studied in detail to better discern phage-induced changes from bacterial responses against phage. The results establish the relevance of using complex settings that mimics *in vivo* conditions to study phage-bacteria interplay, being obvious the phage versatility on bacterial cell invasion.

4.1 Introduction

P. aeruginosa is an opportunistic pathogen responsible for several human infections, being a prevalent colonizer of CF-patients' airways [1]. *P. aeruginosa* infections are often difficult to treat due to their ability to evade host immunity and antibiotics therapy (reviewed in [2]) and, in most cases, the patient's treatment fails. Since *P. aeruginosa* has emerged as a MDR species, these bacteria represent one of the most concerning pathogens that urgently require the development of alternative therapies [3]. The increasing prevalence of MDR bacteria has turned the attention to phages as natural bacterial predators [4,5]. Although the phage therapy concept is over a century old, the impact of phages in bacteria and human cells is not comprehensively studied at the molecular level, leading to doubts concerning its safety and efficacy, which in turn is a major constraint towards its approval as an adjunct therapy to antibiotics treatment [4].

To better elucidate the phage-bacteria interplay at the molecular level, a number of high-throughput technologies in metagenomics, proteomics, metabolomics, and transcriptomics are currently being applied in the phage research field [6–8]. Indeed, massive use of genomics/metagenomics allowed the acquisition of a large number of genomes to better understand phage distribution and evolution in ecosystems and microbiomes [9]; proteomics and metabolomics have enabled the elucidation of complex networks of proteins and metabolites from both phage and bacteria [10–15]; and transcriptomics provided a high content of information about phage-bacteria gene expression at specific stages of the phage life cycle.

Specifically, by using RNA-seq, in addition to unveiling the relevance/function of specific phage genes and interaction partners, mechanisms behind genes regulation, the delineation of transcript boundaries, the discovery of novel antisense genes and the understanding of bacterial expression circuits under phage predation are now attainable [7,8,15–24]. However, while the application of high-resolution RNA-seq-based techniques to the phage field is increasing [17,25,26], the experimental design of these works has not yet addressed the gap in capturing phage-bacteria transcriptional changes during *P. aeruginosa* infection situations. While some models have been applied to simulate *in vivo* lung infections (lungs from CF patients [27], CF sputum samples [28], mouse [29], and zebrafish [30] models), these have not yet been supported by phage-bacteria transcriptome analysis.

In the previous chapter, the *Pseudomonas* LUZ19 phage infection transcriptome was studied during *P. aeruginosa* PAO1 growth in MCCM media (commonly used to grow human airway epithelial cells) as a first step to evaluate growth conditions of *P. aeruginosa* phage infection process on host

physiology. This study supports the current analysis and elucidate the phage's ability to efficiently progress with bacterial infection independently of growth medium, while *P. aeruginosa* revealed major transcriptional differences at early infection related with transcriptional patterns observed in CF bacterial isolates, in anaerobic conditions and biofilm conditions. As phage infection progressed, the transcriptional changes decreased over time, indicating that the phage can adjust the bacterial metabolism towards an efficient infection [16].

Considering the importance of understanding bacterial responses towards phage predation and the drastic differences in bacterial behaviour under host physiology conditions, it is here explored the *P. aeruginosa* transcriptome in a three-way design: phages-bacteria-human cells. For that, it was applied human airway epithelial cells to better mimic phage-bacteria-host interplay, from the microbial perspective.

4.2 Materials and Methods

4.2.1 Bacterial strains, phage propagation, and growth conditions

The reference strain *P. aeruginosa* PAO1 (DSM22644) from The German Collection of Microorganisms and Cell cultures was used in all experiments and was grown at 37°C on Lysogeny Broth (LB) and LB agar (LB with 1.2% (w/v) of agar). Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM:F12) (Gibco) supplemented with 2% (v/v) of Ultrosor-G Serum Substitute Medium (Startorius) was used for synchronized infection assays and human lung epithelial cells cultivation. This medium supports the growth of human airway epithelial cells, and is referred to as MCCM [31]. *Pseudomonas* phage LUZ19 [32] was propagated on *P. aeruginosa* PAO1 cells by standard soft agar overlay, followed by PEG8000 precipitation, and stored in phage buffer pH 7.5 (10mM Tris-HCl, 10mM MgSO₄, 150mM NaCl) at 4°C [33]. Phage titration was performed using the double-agar layer [34].

4.2.2 Airway surface model

The Nuli-1 cell line (ATCC® CRL-4011™) from human airway epithelium of normal genotype patient was kindly provided by Professor Brian Harvey from Department Molecular Medicine in the Royal College of Surgeons (Ireland). Cells were propagated on tissue culture flasks at 37°C, 5% CO₂, and >90% humidity (Heracell 150, Heraeus) using a MCCM medium supplemented with 1% Gibco™ Antibiotic-Antimycotic (100×). When cells reached about 80% confluence, sub-culturing at a 1:3 flask ratio was performed. Next, cells were used to seed six-well tissue culture-treated plates, at 3×10⁵ cells/mL in pre-

warmed MCCM medium (without antibiotic/antimycotic), being incubated at 37°C, with 5% CO₂, and with >90% humidity until reaching confluency. After obtaining a cell monolayer in six-well plates, these were used to perform synchronized infection assays of adhered bacteria with LUZ19 phage (Figure 4.1).

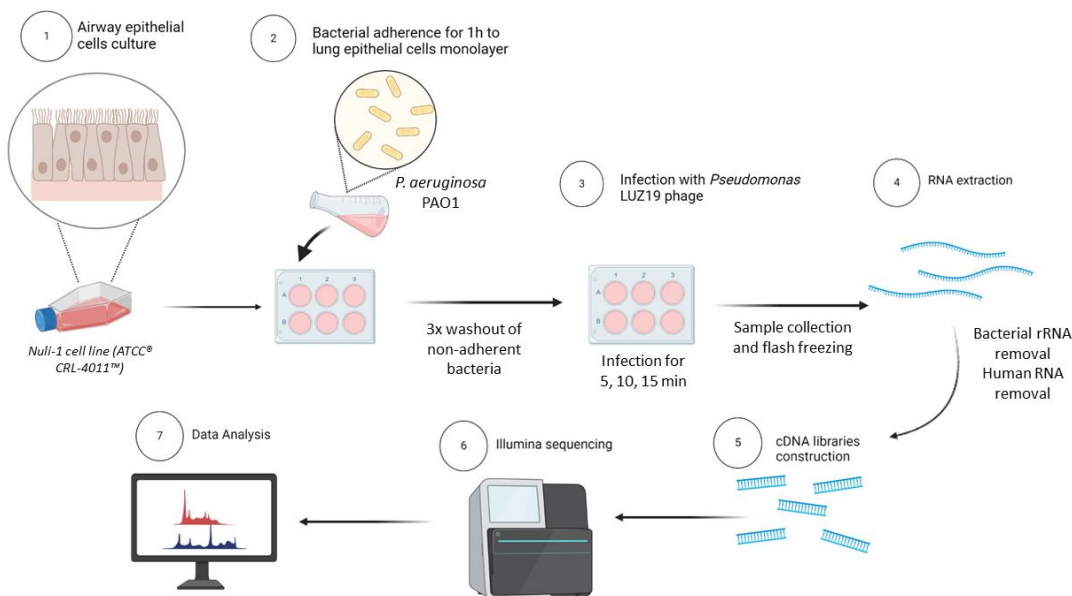


Figure 4.1- Overview of the methodology used for RNA samples acquisition from the complex mixture of Nuli-1 epithelial cells-bacteria-phage, library preparation and Illumina RNA sequencing.

4.2.3 Bacterial adherence to lung epithelial cells monolayer

P. aeruginosa PAO1 overnight cultures were centrifuged (8,000×g, 4°C, 5min), the pellet was washed twice with Phosphate Buffered Saline (PBS: 137mM NaCl, 10mM Sodium Phosphate Dibasic, 2.7mM Potassium Chloride, and Potassium Phosphate Monobasic, pH = 7.4) and the OD_{600nm} was adjusted to 0.13 (approximately 1×10⁸CFU/mL) with MCCM medium. Then, 1mL of bacterial suspension was added to the wells containing NuLi-1 cells and the six-well plates were incubated at 37°C for 1h to allow bacterial cells to adhere to the NuLi-1 cell monolayer.

4.2.4 Synchronized infection of adhered bacteria with LUZ19 phage

Non-adhered bacteria were removed by washing the wells with PBS and in the control plate (bacteria adhered to epithelial monolayer), adhered bacteria (1×10⁷CFU/mL) were collected by mechanical scraping and resuspended in 1:10 vol of an ice-cold stop solution (1:10 buffered phenol, 9:10 absolute EtOH). This sample was flash-frozen in liquid nitrogen to block RNA transcription and degradation until sample processing. In the remaining plates, after removal of non-adhered bacteria by

consecutive washing with PBS, phage LUZ19 was added at high MOI of 75 (to ensure synchronous infection of the culture) in MCCM medium. Plates were incubated for 5, 10, 15, and 20min at 37°C, 5% CO₂. At each time point, the supernatant was removed quickly, and infected bacteria were collected as described in the previous section. Triplicate samples from synchronized infections and control samples (t=0min, uninfected bacteria adhered to epithelial cells monolayer) were used for total RNA extraction.

4.2.5 Total RNA extraction, quantification, quality assessment, and rRNA depletion

Three independent biological assays were performed, collecting a complex mixture of *P. aeruginosa* PAO1 adhered to Nuli-1 cells and infected with LUZ19 phage for 0min (control), 5min (early-phage infection), 10min (middle-phage infection), and 15min (late-phage infection).

Samples stored at -80°C were thawed on ice and centrifuged (3,345×g, 4°C, 20min). Total RNA was extracted from cell pellets using the Purelink RNA Mini Kit (Invitrogen), according to the manufacturer's instructions. Subsequently, each sample was treated with TURBO DNase (Invitrogen) at 37°C to remove genomic DNA (gDNA) and afterwards was co-precipitated overnight at -20°C in 1:10 (v/v) NaOAc and 2.5 vol of absolute EtOH.

Precipitated RNA samples were centrifuged (16,000×g, 4°C, 1h) and pellets were washed twice with 70% (v/v) EtOH. The air-dried RNA pellets were dissolved in 60µl of DNase and RNase-free water (Invitrogen). Total RNA concentration and purity were measured on a Nanodrop 2000 Spectrophotometer (Thermo Scientific, MA, USA) and a Qubit 4 Fluorometer (ThermoFisher Scientific). The absence of gDNA contamination was confirmed by PCR using specific primers for both phage LUZ19 (Forward: 5'-ccgacaagatcgccgcatgc-3', Reverse: 5'-ggctgaagctacgcttgcc-3') and its host (Forward: 5'-cggtcggaatctctacgcacc-3', Reverse: 5'-cgacgccaacaacgccacg-3').

To assess RNA integrity, samples were analysed on a Bioanalyzer (Agilent, Santa Clara, California, US) using a RNA 6000 Nano Kit (Agilent), according to the manufacturer's instructions. Samples with adequate RNA integrity (RNA integrity number (RIN) > 8) were treated with MICROBEnrich™ kit (Invitrogen) to deplete mammalian RNA from the complex total RNA mixtures, according to the manufacturer's instructions.

4.2.6 cDNA libraries preparation and sequencing

Mammalian RNA-depleted samples (n=3 per condition) were directly used to deplete bacterial rRNA and cDNA libraries construction following Ribo-zero (Illumina) manufacturer's recommendations.

Successful rRNA depletion was verified on a Bioanalyzer using the DNA High sensitivity kit (Agilent) and the final concentrations were determined using the Qubit 4 Fluorometer (Invitrogen).

Samples were processed immediately, according to Illumina Stranded Total RNA Prep with Ribo-Zero Plus TruSeq Stranded mRNA Library Prep recommendations. In the end, cDNA libraries were evaluated on a Bioanalyzer (Agilent) to confirm average fragments between the range of 200-300bp, and final concentrations were measured on a Qubit. The libraries' normalization and pooling were performed according to MiniSeq System Denature and Dilute Libraries Guide: protocol A from Illumina. For each run, a library of three samples with equimolar amounts was combined with PhiX control (spike-in of 0.5-2%) and paired-end sequenced (2×75bp) on an in-house Illumina Miniseq sequencer. After a first sequencing run, specific samples were re-sequenced to increase the sequencing depth of the phage and bacterial transcriptomes and improve the statistical power to detect differentially expressed genes.

4.2.7 Data analysis

The quality of the cDNA reads was analysed using FastQC (0.11.8) [35]. Adapters and poor-quality sequences were trimmed using Trimmomatic (v0.39) [36]. Paired-end reads were aligned to the LUZ19 (NC_010326.1), *P. aeruginosa* PAO1 (NC_002516.2), and Human reference genomes (assembly GRCh38.p13) using the BWA-MEM aligner [37] and SAMtools (v1.9) [38] was used to process the alignment files and assess mapping quality. Next, the aligned read pairs (fragments) were assigned to the genomic features of the phage and the host using featureCounts [39]. Alignment visualization was performed using Integrative Genomics Viewer (IGV) [40].

Reads that mapped to the human genome and bacterial rRNA reads (60-80%) were removed *in silico*. Subsequently, fragment counts were first normalized by gene length and next by the number of non-ribosomal read pairs in each sample to obtain FPKM (fragments per kilo base of transcript per million mapped fragments) values. Samples variance was analysed using PCA in R. Differential gene expression analysis was performed using a negative binomial distribution test from the DESeq2 Bioconductor package in R [41]. An ANOVA test was performed to identify the most significantly up or downregulated genes.

The bacterial transcriptome during LUZ19 phage infection when adhered to human airway epithelial cells was analysed to elucidate bacterial major responses triggered by the phage under human physiological conditions. Sequencing of the RNA samples revealed that, despite the depletion of ribosomal RNA (rRNA) and mammalian RNA, a high proportion of reads mapped to the human genome and rRNA, (Table S4.1 and Figure S4.1). The reduced sequencing depth of the bacterial transcriptome constitutes

a limitation of using the triple RNA-seq technique where most of the reads map to the mammalian host cells, which can be overcome by using high-throughput sequencers systems. Considering this technical limitation, to increase the number of reads that map to the bacterial genome, it was re-sequenced three samples (4_5, 7_5, 5_10, and 4_15) to achieve at least $\approx 500,000$ prokaryotic non-rRNA reads per sample, sufficient for reliable data analysis. The data that support the findings of this study are deposited into GEO database with an access number GSE213159.

4.3 Results

4.3.1 *Pseudomonas* LUZ19 phage transcriptome is consistent for standard LB growth medium, mammalian cells culture medium, and lung epithelium presence

The phage transcriptional landscape during infection of bacteria adhered to epithelial cells is very similar to the LUZ19 transcriptomes previously observed in LB or MCCM media [16]. More specifically during infection of bacteria adhered to epithelial cells monolayer at 5min-post infection, the reads were mapped at regions located in the beginning of phage genome (position 0 > $\approx 10,000$ bp), then at 10min-post infection reads were also mapped at beginning and middle regions of phage genome (0 > $\approx 25,000$ bp), and at 15min-post infection a high number of reads were already mapped also in regions located closer to the end of the phage genome (25,000 > 45,000bp), as the infection progresses, as visualized in Figure 4.2. Under these conditions, the number of reads was significantly reduced (Figure S4.2) as a consequence of performing triple RNA-seq. In addition, the burst size was reduced (Figure S4.3) because of a lower bacterial cell growth rate in these conditions.

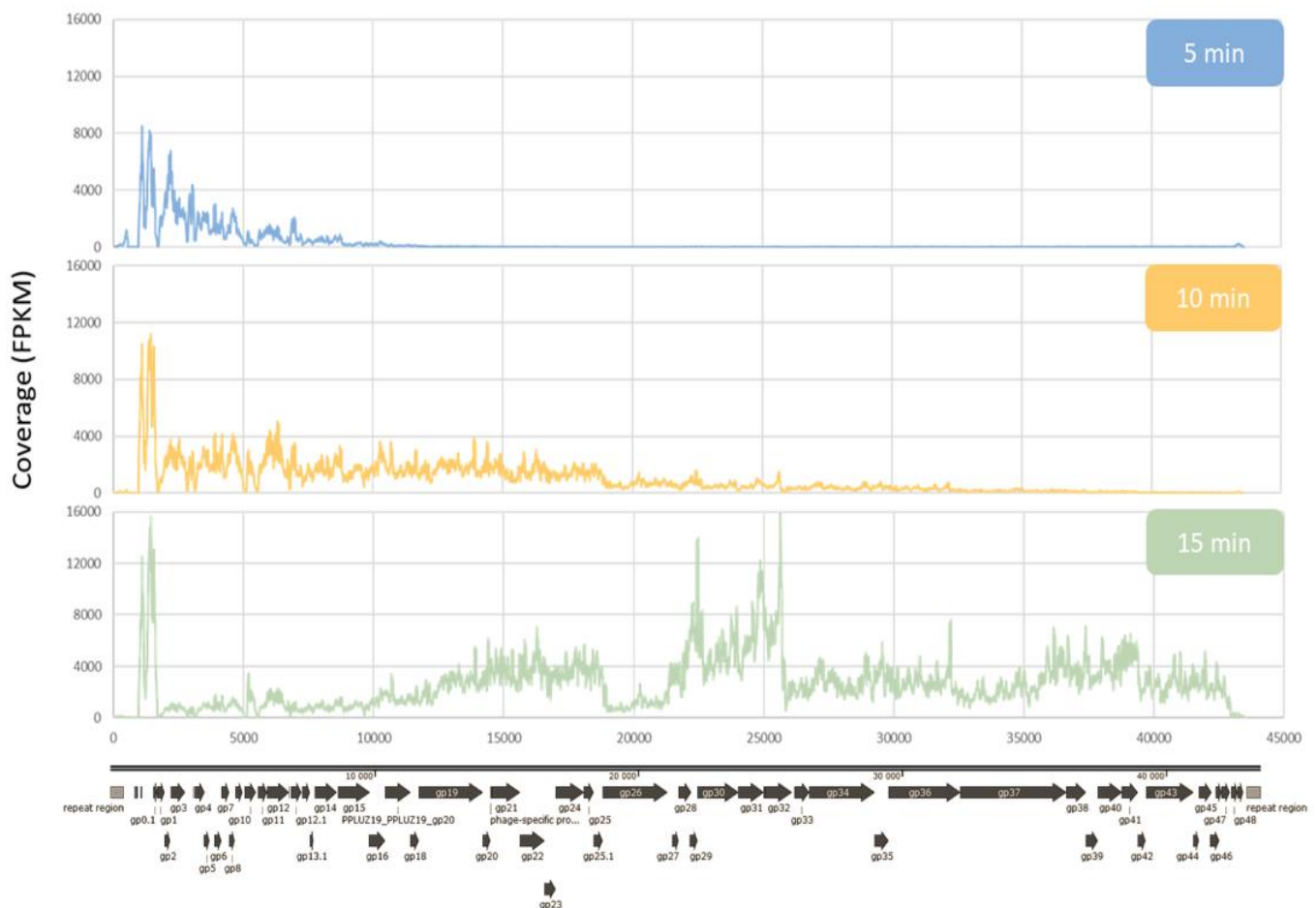


Figure 4.2- *Pseudomonas* phage LUZ19 transcriptional landscape on *P. aeruginosa* PAO1 adhered to Nuli-1 epithelial cells. 5min samples, 10 in samples and 15min samples represent the phage transcripts at the early middle, and late infection stage, respectively.

Considering similarities between expression patterns obtained in LB, MCCM [16] and in the conditions reported in this study, a PCA analysis was performed (Figure 4.3) of the overall transcriptomes. Before phage exposure (t=0min) the bacteria have very distinct gene expression patterns when comparing the different media conditions, yet the presence of Nuli-1 epithelial cells introduces a variable that leads to a very distinct transcriptome from both MCCM medium and LB medium. Looking closer to the samples that are under phage exposure, the transcriptomes in different media begin to converge over time until the end of the infection. Overall, phage transcription mechanisms seem to proceed efficiently with minimal changes.

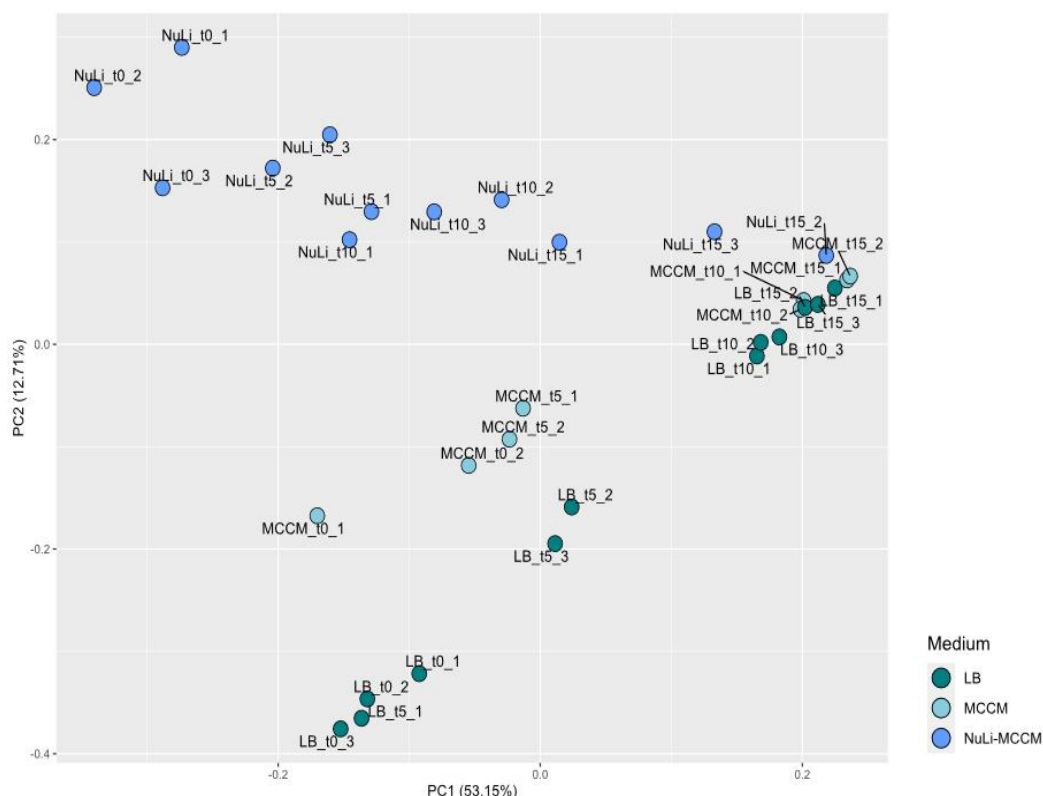


Figure 4.3- Principal Component Analysis of total transcriptomes from samples acquired at early (5min), middle (10min), and late infection (15min) in the presence of LB media, MCCM media, and Nuli-1 epithelial cells. The control samples (without phage presence) in each respective media are also included (t0). (Figure contains data described in chapter III).

4.3.2 *P. aeruginosa* PAO1 Differential Expressed Genes (DEGs) during phage infection in the presence of Nuli-1 epithelial cells

The transcriptome of *P. aeruginosa* PAO1 in the presence of Nuli-1 epithelial cells was analysed during phage infection and compared to the transcriptome of uninfected bacteria. Thresholds used to find DEGs were placed at $-1.5 \leq \log_2 \text{Fold} \leq 1.5$, and $\text{padj} \leq 0.05$. Under these conditions, 21 DEGs were obtained 5min post infection, 39 DEGs at 10min, and 129 DEGs at 15min, as shown in Figure S4.2 and Figure 4.4. Generally, DEGs upregulated during early infection were associated with lipid A modification, glycerol metabolism, translation, the prokaryotic degradosome, and phosphate acquisition, whereas aromatic amino acids transport DEGs were downregulated. At 10min, genes involved in sulphate transport, phosphate transport, spermidine biosynthesis, glycine metabolism, pyochelin biosynthesis, and protein folding are upregulated. By contrast, the aromatic amino acids transport, arginine/ornithine transport, lipid A modification, lactate metabolism, and several transcriptional regulators are relatively

downregulated at 10min post infection. In the late infection stage, a larger number of differentially transcribed genes were upregulated: genes involved in sulphate transport and sulphur metabolism, spermidine biosynthesis, fatty acid biosynthesis, pyoverdine biosynthesis, metabolism of specific amino acids (glycine, serine), transcription, protein folding, ribosomal proteins and RNA, taurine metabolism, phosphate transport, EPS biosynthesis, pyochelin biosynthesis, two-component systems, and antibiotic precursors (β -lactamase, isopenicillin N); and downregulated: genes associated with amino acids transport (branched amino acids, acidic amino acids), amino acids metabolism (tyrosine, glutamine, isoleucine/leucine, valine, histidine), carbon metabolism, lactate oxidation, oxidative phosphorylation, acetyl-coA metabolism, LPS synthesis, type IV pili synthesis, and virulence transcriptional regulators.

To understand if the transcriptional responses obtained from RNA-seq of samples acquired during lung epithelial cells exposure are specific to Nuli-1 cells growth condition or if they are caused only by the phage regardless of the bacterial growth conditions, the data was compared with the previously published bacterial transcriptomes in LB medium and MCCM medium [16]. In total, 17 genes were found to be differentially expressed in all growth conditions, 78 genes are specifically differentially transcribed in the Nuli-1 condition, 242 are specific for LB condition, and 721 for MCCM (Figure 4.5). Interestingly, among the 17 genes that are differentially expressed in all media, four are annotated as probable phage proteins, although their role during phage infection is unknown. Besides phage-related genes, genes involved in glycine cleavage, spermidine biosynthesis, post-translational modification (tpbA), tyrosine catabolism, glutathione metabolism, and leucine catabolism were also found to be commonly differentially transcribed in all growth conditions (Table S4.3). Genes exclusively differentially transcribed in the Nuli-1 condition can be seen in Table S4.4.

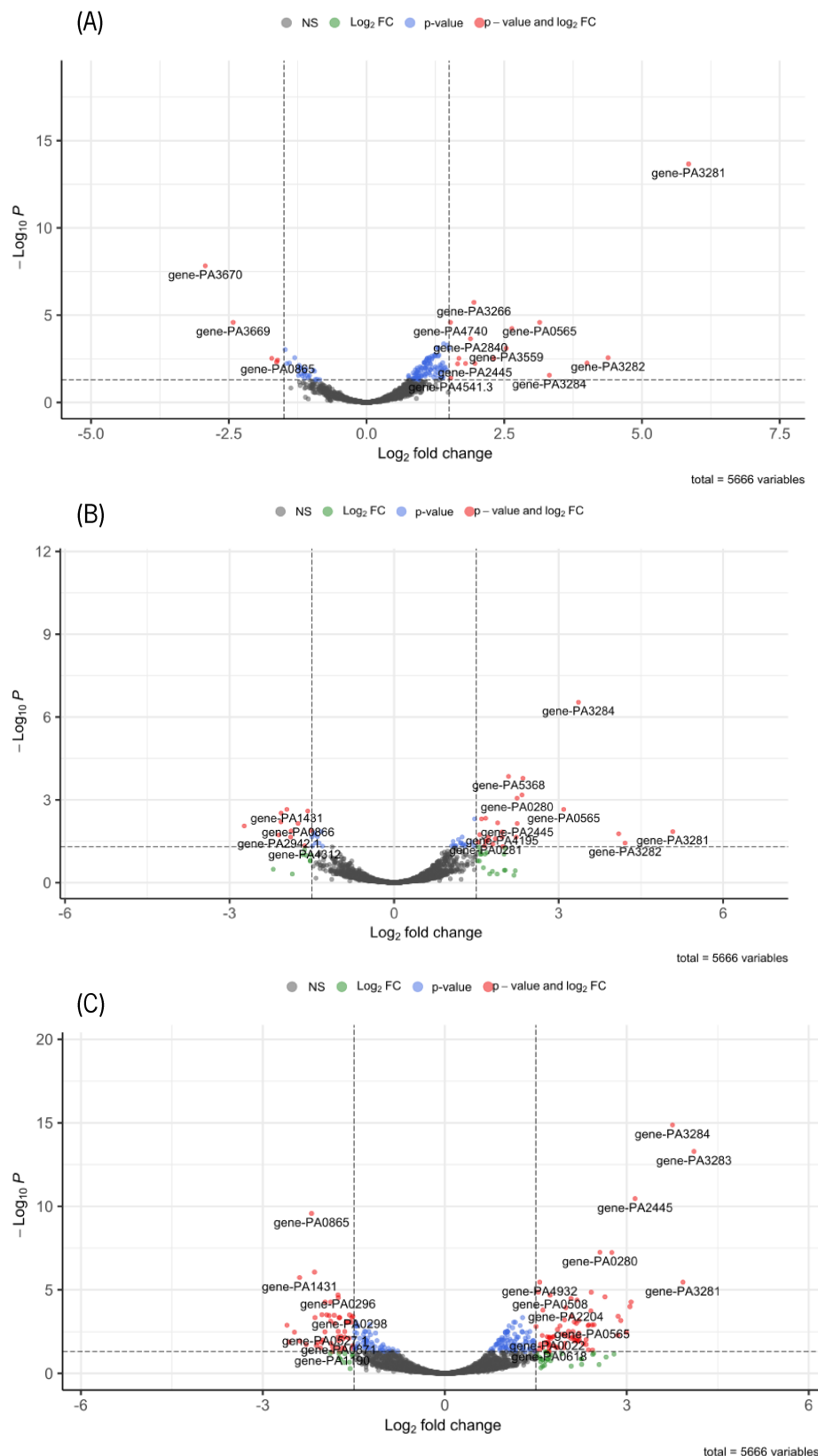


Figure 4.4- Volcano plots representing *P. aeruginosa* PA01 DEGs after 5min (A), 10min (B), and 15min (C) of infection with LUZ19 phage ($-1.5 \leq \log_2 \text{Fold change} \leq 1.5$, and $\text{padj} \leq 0.05$).

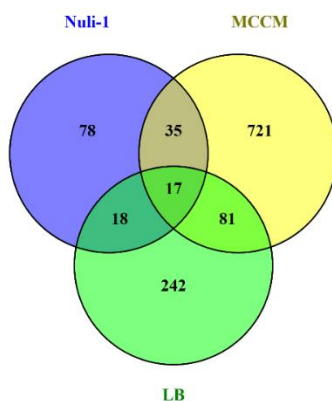


Figure 4.5- Venn diagram showing genes that are exclusively differentially transcribed in each media and the genes that are commonly expressed in each condition. The intersection of each condition represents the number of shared genes that are differentially expressed.

4.3.3 Conserved induced phage responses between the different bacterial growth conditions

To understand the transcriptional changes that occurred in the different growth conditions during the phage infection, it was combined previously obtained complementary data from Chapter III [16] with new data reported here and represented the Log2Fold values over time (5, 10, 15min) as a heat map (Figure 4.6). The first common transcriptional changes that appear to be triggered during phage infection regardless of the growth conditions are the genes related to spermidine biosynthesis. *spE2* is among the most significant differentially expressed genes. This gene is significantly upregulated in MCCM and Nuli-1 conditions and is downregulated in LB. Another consistently triggered transcriptional change was related with the increasing upregulation of hypothetical phage proteins and hypothetical prophage Pf1 genes in all bacterial growth conditions as phage infection progresses. On the other hand, the opposite is observed for the operon that comprises genes *hisF2*, *hisH2*, *wzx*, *wzy*. These genes are involved in the biosynthesis of the heteropolymer o-specific B-band O-antigen of LPS, and are increasingly downregulated over time in all growth conditions. In addition, during the early stage of phage infection and specifically on bacteria adhered to lung epithelium and grown on MCCM, glycerol metabolism is induced, although upregulation of genes associated with this metabolism becomes less pronounced over time. Lastly, alginate-associated biosynthetic genes are also triggered after phage infection. Whereas the alginate-related genes are upregulated in the presence of LB medium and Nuli-1 epithelium, it was observed a drastic shift from downregulation to upregulation in MCCM medium (with the exception of gene *algT*/transcriptional regulator). The transcriptional profile for type IV pili and flagella-associated genes is less pronounced,

although in general, these appear to be upregulated during early infection (for MCCM and Nuli-1 conditions) and subsequently become less transcribed over the course of infection.

In all growth conditions, *rsaL* (control of QS cascade) and *dpsI* (virulence-related) genes also appear to be common phage targets, as they are downregulated over the course of infection, with exception of *dpsI* in LB medium.

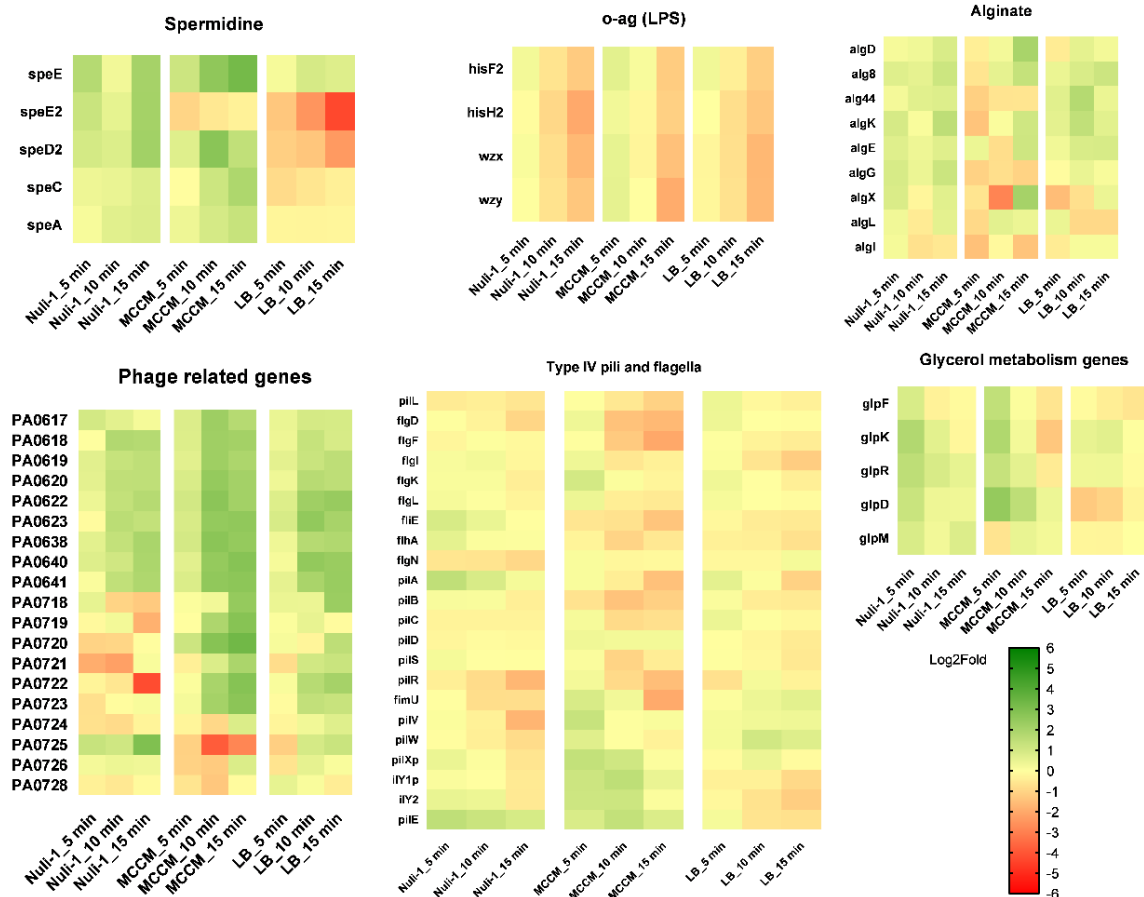


Figure 4.6- Heat maps of genes Log2Fold values for each categorical group of genes at different stages of infection (5-, 10-, and 15min post-infection) in bacteria adhered to Nuli-1 epithelial cells monolayer, grown on MCCM media, and grown on LB media. Log2Fold values are always relative to the expression level of uninfected bacteria at each respective growth condition.

4.3.4 Specific phage induced responses in the presence of lung epithelial cells

Even though the phage efficiently progresses with its infection and leads to major changes in the bacterial transcriptome, which seem to be common for all tested media, some specific transcriptional changes unique to the lung epithelial cell condition were observed (Figure 4.7). Among these differences,

the upregulation of sulphate-related genes over the infection time was observed only for the Nuli-1 condition, which indicates a requirement of this nutrient during phage infection, perhaps by providing a phage fitness advantage under this condition [42,43]. In addition, the transcription of the *arn* operon, which is associated with the modification of the lipid A component of LPS, is upregulated during infection when bacteria adhere to Nuli-1 epithelial cells. Likewise, the genes that positively regulate the *arn* operon, the *pmrA*/*pmrB* belonging to the two-component system, are also upregulated during infection.

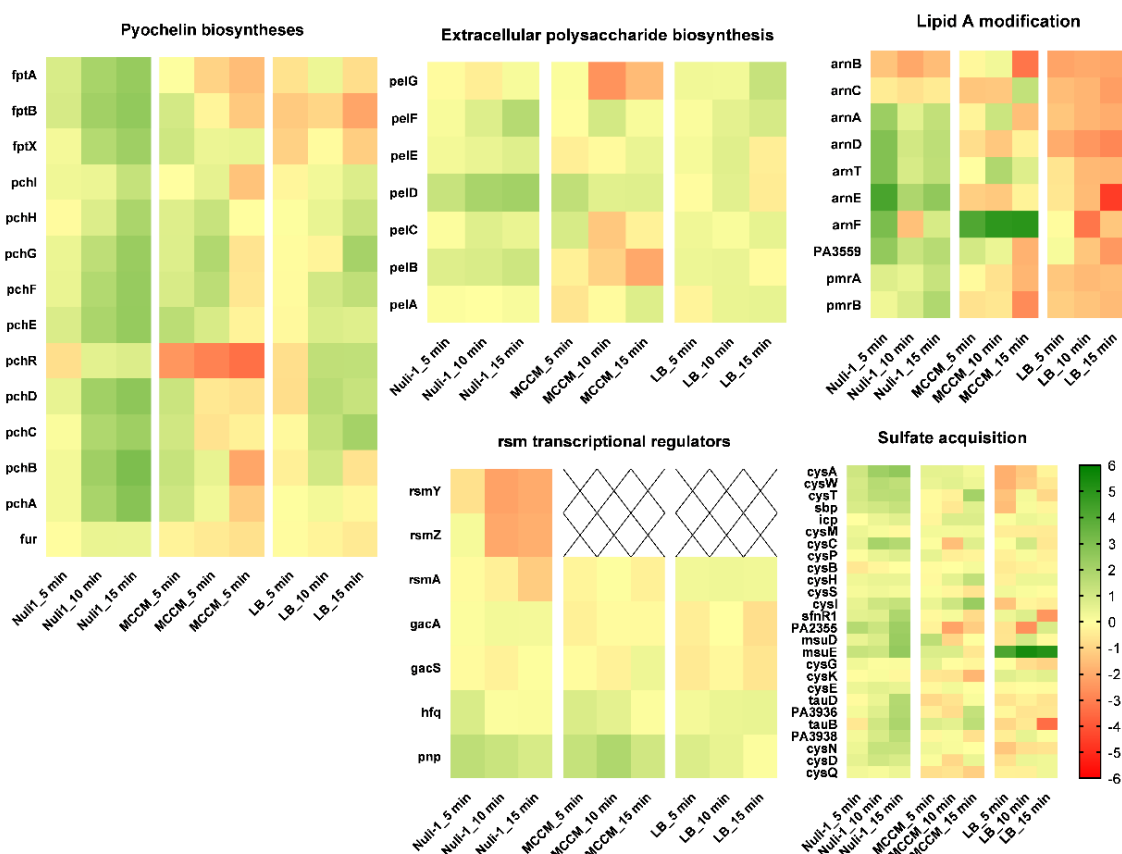


Figure 4.7- Heat maps with Log2Fold values for each group of genes that are specifically targeted at Nuli-1 at different stages of infection (5-, 10-, and 15min post-infection) in bacteria adhered to Nuli-1 epithelial cells monolayer, grown on MCCM media, and grown on LB media. Log2Fold values are always relative to the expression level of uninfected bacteria at each respective growth condition.

Siderophore pyochelin synthesis-associated genes are also significantly differentially expressed in the Nuli-1 condition during phage infection, as well as in LB medium, although slightly attenuated compared to the Nuli-1 environment. Considering the importance of iron homeostasis in *P. aeruginosa*, the expression of genes involved in iron acquisition was evaluated in the three growth conditions (Figure S4.4). In the Nuli-1 condition the overall set of iron-related genes do not seem to be differentially

transcribed with the exception of pyochelin. By contrast, iron-related genes are generally upregulated in LB and a strong downregulation is observed in the MCCM medium, with exception of *bfd* (bacterioferritin-associated ferredoxin). To better understand these differences between media, the transcription of the same genes at each medium without phage exposure was also examined. Comparison between the different conditions showed that bacteria are highly transcribing iron-related genes in the MCCM medium relative to the Nuli-1 and LB growth conditions, and the same genes are upregulated in the Nuli-1 condition compared to LB medium. Therefore, bacteria are increasing expression of these genes in the following sequence: MCCM>Nuli-1>LB, indicating that the bacteria are showing the most iron starvation in MCCM, followed by the Nuli-1 condition.

Another interesting transcriptomic modulation during phage infection of bacteria adhered to lung epithelium is the consistent upregulation of genes involved in T3SS, which in LB and MCCM seem to be downregulated during infection, relative to uninfected bacteria. Also, non-coding RNAs (ncRNAs) *rsmY* and *rsmZ* were found to be downregulated during infection when bacteria is adhered to Nuli-1 cells. The Log2Fold values of their transcriptional regulators *gacA/gacS*, as well as *rsmYZ*'s final target (*rsmA*) and other genes such as *hfq* and *pnp* involved in *rsmYZ* stability and degradation were also evaluated. The results demonstrated that *pnp* is upregulated during infection, especially in Nuli-1 and MCCM, and that *hfq* expression is reduced over the course of infection.

RsmA, a global virulence regulator, is exclusively downregulated in the Nuli-1 condition, although its transcriptional regulators (*rsmY* and *rsmZ*), which are both downregulated throughout infection. On the other side, *gacA/gacS* seem to not be differentially expressed during infection, which is likely related to the non-transcription of *rsmY* and *rsmZ*. In addition, *pnp* which encodes an PNPase and is involved in the *rsmY* and *rsmZ* degradation, is upregulated in both MCCM and Nuli-1 conditions. This might explain the decay in *rsmYZ* transcripts since *hfq* (involved in *rsmYZ* stabilization) is also decreasing over the infection, especially in Nuli-1 condition. As a final remark, another Nuli-1 specific transcriptional change was observed for the gene *mvaT*, which is increasingly downregulated over the infection.

4.4 Discussion

Unveiling phage-bacteria interplay under conditions that mimic human physiology is a crucial step in phage biology research. Previously, it was demonstrated that bacterial media conditions significantly impact the phage-bacteria transcriptome [16]. Here, it was increased the design complexity by tracking transcriptional changes under conditions that more closely mimic the human lung epithelium

conditions. It was observed that the phage was able to infect adhered bacteria and that transcribes its genome following the same temporal gene transcription as revealed in other media (LB and MCCM medium). As previously observed, the bacterial growth rate appears to influence phage burst size and only a limited effect on phage transcription pace was detected. By contrast, significant differences in the bacterial transcriptome were observed, despite the lower sequencing depth obtained when combining phage/bacteria/human RNAs. An overview of important changes can be observed in Figure 4.8.

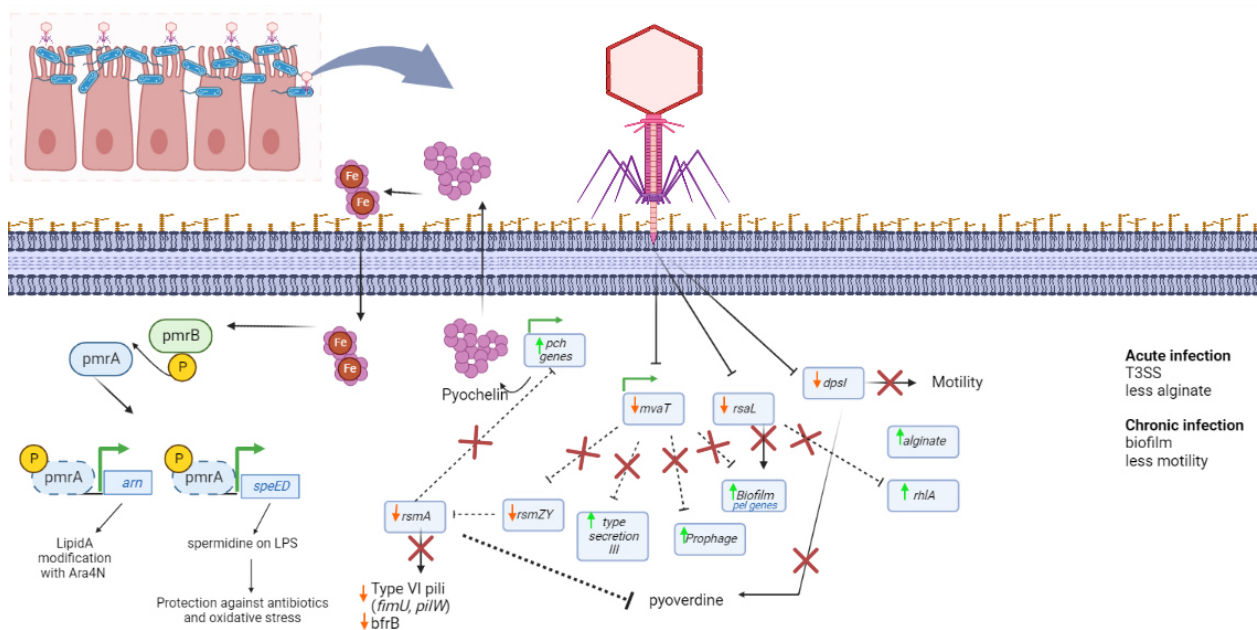


Figure 4.8- Main overview of transcriptional changes induced on bacteria by phage during infection in the presence of human lung epithelial cells.

4.4.1 Understanding the “Core” strategy of phage predation

In the presence airway epithelial cells, LUZ19-induced transcriptional differences in the bacteria can be inferred to be part of a common predatory strategy. One of the most reported responses induced during *P. aeruginosa* phages infection is the upregulation of probable phage proteins and hypothetical prophage genes [7,8], which has been suggested to be a mechanism unleashed by the host stress response towards phage predation, leading to prophage escape. By using different growth conditions, this data confirmed that these results remain constant and indicate a conserved phage-bacteria interaction regardless of the growth environment.

From the main changes observed in the bacterial transcriptome during LUZ19 infection in lung epithelium presence, a general response on bacterial surface receptors concealment was observed, which was also observed in LB and MCCM growth conditions. For instance, the operon that comprises

genes *hisF2*, *hisH2*, *wzx*, and *wzy*, involved in the biosynthesis of the heteropolymeric O-antigen (O-Ag) polysaccharide from LPS is commonly downregulated during infection, a phenomenon also observed during ϕ KZ phage infection [17]. Hypothetically, this can be an approach for bacteria to acquire phage resistance or, a phage prompted action to prevent secondary infections [44]. In addition to inhibition of LPS synthesis, bacteria also express genes associated with alginate production during infection. If bacteria increase alginate production, this can ultimately result in bacterial receptor's protection against phage infection. Nevertheless, alginate gene upregulation might be induced due to the stress imposed by phage infection on the cell since that alginate production is usually associated with higher bacterial tolerance to stress [45].

Consistently, the transcription of flagella and type IV pili were downregulated in all growth conditions, which has been also observed for several other *P. aeruginosa* phages infections. This downregulation is usually mediated by phage-proteins as a strategy to avoid bacterial superinfection and reduction of bacterial motility [18,46]. Therefore, regardless of bacterial physiological state, functions related to bacterial receptors concealment are promoted during phage predation, even under lung mimicking conditions. In the future, it might be important to understand if this induced bacterial surface modification is part of a phage-induced mechanism to avoid cell superinfection or a bacterial mechanism to resist to phage.

4.4.2 Phage triggers alternative energy/substrates sources during infection in the presence of lung epithelium

Under more complex conditions, specific bacterial transcriptional differences can be observed. First, as previously reported on MCCM media, genes involved in glycerol metabolism are being specifically transcribed on phage early infection for this condition [16] but also under Nuli-1 epithelial cells presence. This metabolism is important during *P. aeruginosa* colonization of airway sputum, as glycerol functions as an essential nutrient and appears to be involved as a precursor for virulence factor production [47]. The data revealed that under lung mimicking conditions, the phage triggers this alternative pathway to acquire energy to attain optimal infection conditions. In addition, phage infection leads to a “sulphate starvation-like condition”. The usurpation of sulphate metabolism can be beneficial to the phage by increasing its fitness [43], especially when this substrate is in abundance, which seems to be the case under the presence of epithelial cells that can produce mucins, an important source of sulphate for *P. aeruginosa* under contact with host cells [48].

4.4.3 Under lung physiology conditions the phage employs hidden predation mechanisms by inducing LPS modification and pyochelin synthesis

A phage-prompted transcriptional answer that was observed only in MCCM medium and under the presence of lung epithelial cells was the upregulation of spermidine biosynthesis. Spermidine is a polyamine pointed to be a very important substrate for efficient phage infection [49], playing a role in phage DNA stabilization and compaction by neutralizing the negatively charged phage genomes [50,51]. Indeed, polyamines seem to play an important role in the viral relationship between host cells and their metabolites, including viruses that infect bacteria, plants, and mammals [52]. Recently, polyamines have been identified as a new phage defence mechanism, where polyamines liberated from phage-lysed bacteria work as a danger signal to neighbour cells to prevent bacterial death mediated by phage, as this process is mediated by Gac/Rsm signalling. However, this mechanism might be counteracted by LUZ19-encoded proteins since the increase in polyamines doesn't lead to inhibition of LUZ19 phage replication/transcription, and according to this data Gac/Rsm signalling is being downregulated during infection (compared to non-infected bacteria).

A distinct bacterial response was also captured specifically under epithelial cells presence, i.e., the upregulation of genes related to Lipid A modification with *ara4N* and its transcriptional regulators *pmrA/pmrB*. Usually, the upregulation of this operon is related to LPS masking, to protect *P. aeruginosa* from the immune system or for the acquisition of antibiotic resistance against polymyxin B [53]. Curiously, PmrA/PmrB are also described to induce the expression of *speDE* genes as a response to low Mg^{2+} or the presence of extracellular DNA, protecting cells from oxidative damage and antibiotics by binding spermidine to LPS [54]. Therefore, under *in vivo* conditions, phages might activate additional mechanisms to protect the infected cell from other viruses' attacks and probably, even the host immune system, as PmrA/PmrB sensing regulators are an important target under these conditions. This mechanism is usually triggered by PmrB when it senses an increase of periplasmatic iron or a low amount of Mg^{2+} and phosphorylates PmrA that in turn activates the expression of *arn* genes [55]. In addition, iron bounded to PQS or siderophores on periplasm can also induce *arn* operon expression [54].

Considering that iron homeostasis is critically important for *P. aeruginosa* survival, virulence, and pathogenicity [56,57], but also in the phage life cycle [58–60], and since the data indicates strong upregulation of pyochelin siderophore under airway epithelial cells, it was tracked the expression levels of iron associated genes, although genes associated with iron acquisition (with exception of pyochelin) are poorly differentially expressed under these conditions. This is not the case in MCCM media nor in LB

media, in which iron-associated genes are strongly downregulated and slightly upregulated over the infection, respectively. Pyochelin upregulation during phage infection was already observed during phage PAK_P4 infection, as well as other iron acquisition associated genes including pyoverdine and heme acquisition associated genes [23]. This reveals that upon infection, iron metabolism (that is usually tightly regulated in bacteria) is completely deregulated, i.e., without phage exposure the bacteria are in iron-starvation (MCCM > Nuli-1 > LB). Under phage exposure, it would be expected that genes associated with iron metabolism would be significantly upregulated because the production of new virions demands high iron pools that are incorporated in phage tails and enzyme production. Strangely the opposite appears to occur, as genes are downregulated in the media that have a low concentrations of iron (MCCM) and slightly upregulated in LB (higher amount of iron). Surprisingly, in the Nuli-1 condition only pyochelin is significantly upregulated but not pyoverdine. Usually, these two siderophores are induced together under iron starvation. Furthermore, the siderophore that has a higher affinity to iron is pyoverdine [29,61], meaning that in more realistic conditions iron recruitment is not necessary during phage infection, as the upregulation of pyochelin is an unidentified mechanism with an unknown role during phage infection. This observation contradicts previous observations on standard bacterial growth media that usually report that iron is recruited for new viral particles production during phage predation and is crucial during the lytic cycle [59]. Lastly, an alternative iron acquisition mechanism that possibly contributes to the iron pool is the upregulation of Pf phage (it is upregulated in all growth conditions as referred before), since Pf was already described to function as an iron chelator [58]. During infection, this might be an alternative route to assure iron availability inside of the cell instead of using standard mechanisms such as the expression of genes like pyoverdine. Nevertheless, the iron levels increase due to the Pf chelation-associated mechanism does not explain by itself the increasing expression of PmrA/PmrB and consequentially transcription activation or *arn* operon, which is specific to Nuli-1 epithelial cells presence. In the future, it would be worth studying the relationship between the expression of iron, spermidine, lipid A, and prophage associated genes during phage infection since they seem to be connected, especially in lung epithelial conditions.

4.4.4 Shutdown of bacterial global virulence regulators might lead to successful phage infection under realistic conditions

Data from bacteria adhered to lung epithelial cells allowed the observation of downregulation of ncRNAs RsmY and RsmZ. These ncRNAs are responsible for sequestering of global virulence post-translational regulator, RsmA [62]. RsmA modulates T3SS, T6SS, biofilm formation, QS, and siderophores

production [63,64]. The transcription of the *rsmA* gene, transcriptional regulators GacA/GacS (which control *rsmY* and *rsmZ* expression [65]), *prp* gene (degrades *rsmY* and *rsmZ* [66,67]), and *hfq* (protect *rsmY* and *rsmZ* from PNPase degradation [64]) appear to be well correlated between the different growth conditions that were used. However, apart from other growth conditions, Nuli-1 epithelium data reveals that low levels of *rsmYZ* RNAs might be a consequence of its degradation by PNPase (upregulated) or by its transcription inhibition by *pmrA*/*pmrB* (upregulated) [68].

In addition, performing RNA-seq on infected bacteria adhered to epithelial cells enabled the capture a significant downregulation of the *mvaT* (*P16*) and *P15* genes, of which the former encodes a global virulence transcriptional regulator that binds to AT-rich sequences and silences genes [69–71]. MvaT is reported to repress the ncRNAs *rsmY/rsmZ*, but is also involved in the repression of biofilm formation, T3SS, and prophage-associated genes [69–71]. By a specific mechanism that occurs only in lung epithelium, the phage strongly downregulates *mvaT*, which might result in the increase in T3SS expression that it was observed specifically on the Nuli-1 condition. T3SS-secretion system secretes virulence factors expressed by *P. aeruginosa* in acute infections to promote its pathogenicity by delivering a group of effectors in the host cell [72]. On one side, the T3SS system might be increasing bacterial pathogenicity and virulence towards cell exposure to both phage predation and *in vivo* conditions, on the other side, it can be a result of *mvaT* downregulation during infection. Indeed, bacterial MvaT was already described to be targeted by the Mip protein, encoded by *Pseudomonas* phage LUZ24. The mentioned phage protein binds to MvaT and blocks it, impeding MvaT binding to AT-rich sequence portions on phage genomes, therefore avoiding MvaT-mediated silencing of phage genes [73].

In general, many of the significant changes observed in the Nuli-1 condition seem to be interlinked and described in *P. aeruginosa*, although these haven't been connected and elucidated for phages. This justifies the importance of performing assays that use similar conditions to *in vivo* because under conventional LB medium these pathways might not be induced.

4.5 Conclusions

In general, performing RNA-seq on phage-infected bacteria under complex growth conditions is attainable and proves that overall transcriptional interactions can be observed using these assays. This experimental design also highlights how adaptable the *P. aeruginosa* metabolism is, as the bacterium's transcriptome is very distinct for the different growth conditions tested. In parallel, it becomes obvious how robust the hijacking mechanisms of the phage are, in terms of manipulating the bacterial host

metabolism, despite critical differences at the onset of infection. However, the distinction between lab and real conditions influence in predatory mechanisms remains relevant and is likely phage-specific and should therefore be carefully considered in phage-bacteria interactions studies.

4.6 References

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4.7 Supplementary data

Table S4.1- Absolute values of reads mapping for each sample of *P. aeruginosa* infected with LUZ19 phage under the presence of Nuli-1 epithelial cells. Samples, where it was made a second sequencing to increase the depth power, are highlighted in bold.

Sample	Sample ID	Total reads	Reads mapped to PAO1	Reads mapped to PAO1 w/o rRNA	Reads mapped to LUZ19
5-B	t0 NuLi 1	22,655,247	1,164,875	587,178	44
6-B	t0 NuLi 2	21,143,209	1,758,163	841,096	28
7-B	t0 NuLi 3	25,952,330	2,400,326	1,113,004	47
4-5 merged	t5 NuLi 1	34,036,980	1,143,479	589,164	71,559
5-5	t5 NuLi 2	26,876,170	2,604,199	1,459,406	90,603
7-5 merged	t5 NuLi 3	33,455,113	1,390,562	462,542	15,831

2-10	t10 NuLi 1	23,001,713	1,285,799	846,118	139,799
5-10 merged	t10 NuLi 2	32,195,544	1,278,064	797,544	375,499
7-10	t10 NuLi 3	24,434,280	2,492,432	1,032,460	673,179
2-15	t15 NuLi 1	18,940,027	929,769	664,378	849,826
4-15 merged	t15 NuLi 2	34,363,784	1,114,105	560,916	5,737,561
5-15	t15 NuLi 3	22,639,034	1,068,012	708,182	2,086,045

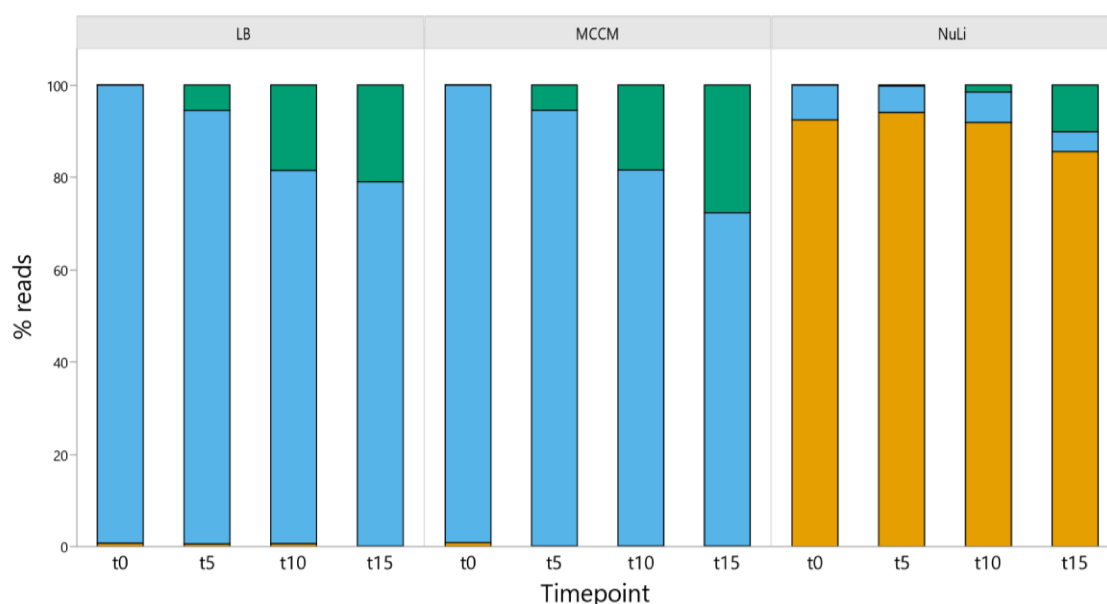


Figure S4.1- Percentage of reads mapping to *P. aeruginosa* PA01 adhered to Nuli-1 epithelial cells and *Pseudomonas* LUZ19 phage in each sample acquired at 0 (control-uninfected bacteria), 5-, 10-, and 15-min post-infection.

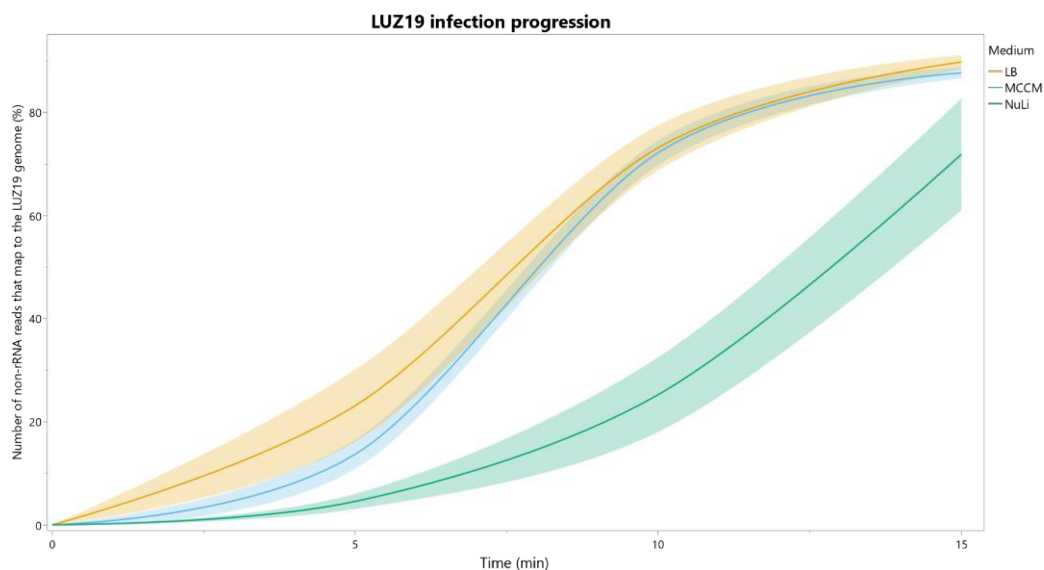


Figure S4.2- Phage LUZ19 transcriptional landscape over the time of infection of *P. aeruginosa* PAO1 grown in LB medium, MCCM, and Nuli-1 epithelial cells.

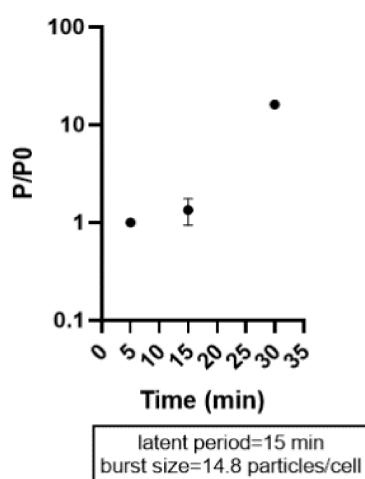


Figure S4.3- *Pseudomonas* LUZ19 one-step growth curve performed on *P. aeruginosa* PAO1 adhered to Nuli-1 epithelial cells monolayer.

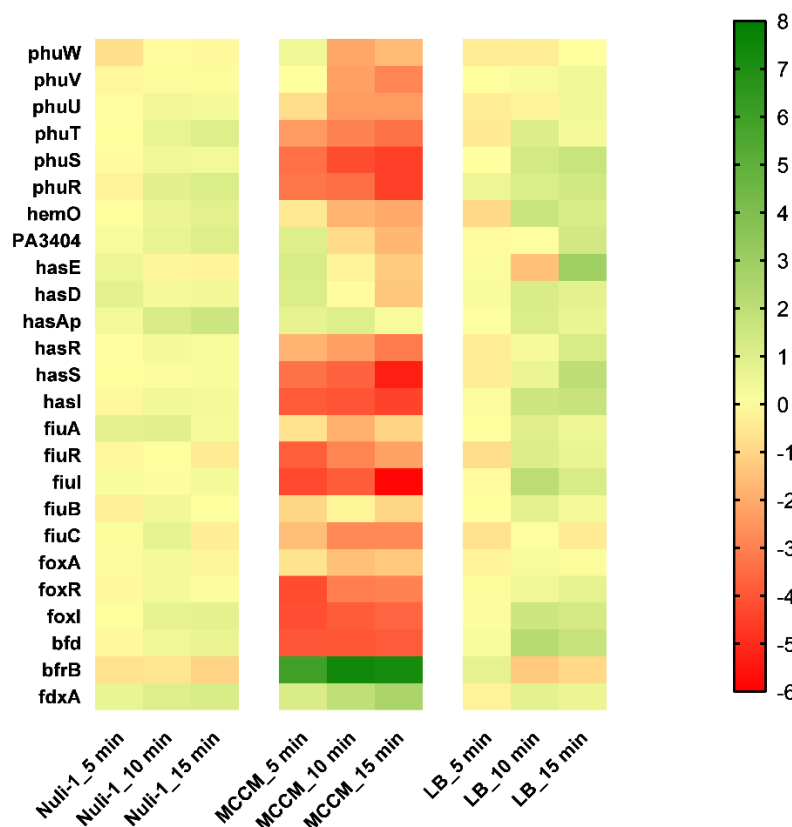


Figure S4.4- Heat map representing the expression of iron-related genes (Log2Fold values relative to uninfected bacteria) during each stage of phage infection, under the presence of different growth conditions.

Table S4.2- DEG of *P. aeruginosa* PAO1 adhered to Nuli-1 epithelial cells monolayer during phage LUZ19 infection at different stages of infection using as control uninfected bacteria.

Locus Tag	Log2Fold	padj	Name	Biological role
Nuli-1 (5min vs 0min)				
PA0865	-1.61	3.19E-03	<i>hpd</i>	tyrosine catabolism
PA0866	-1.63	4.07E-03	<i>aroP2</i>	aromatic amino acid transport
PA2445	1.97	5.45E-03	<i>gcvP2</i>	glycine cleavage system
PA3049	-1.72	2.48E-03	<i>rmf</i>	ribosome modulation factor
PA3266	1.95	5.32E-07	<i>capB</i>	cold acclimation protein B
PA3280	2.64	2.21E-05	<i>oprO</i>	Pyrophosphate-specific outer membrane porin OprO precursor
PA3554	2.31	2.30E-03	<i>arnA</i>	beta-L-Ara4N-lipid A biosynthetic process

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PA3582	1.80	5.45E-03	<i>glpK</i>	glycerol metabolism
PA4541.3	1.53	4.06E-02	tRNA-Asn	Aminoacyl-tRNA biosynthesis and tRNA charging
PA4740	1.52	1.31E-05	<i>pnp</i>	prokaryotic degradosome
Nuli-1 (10min vs0 min)				
PA0280	2.24	1.79E-04	<i>cysW</i>	sulphate transport
PA0281	1.67	1.98E-02	<i>cysA</i>	sulphate transport
PA0866	-1.75	2.08E-03	<i>aroP2</i>	aromatic amino acid transport
PA1431	-1.96	5.54E-04	<i>rsaL</i>	regulatory protein
PA1687	1.70	1.45E-02	<i>speE</i>	spermidine biosynthetic process
PA2445	2.24	3.00E-03	<i>gcvP2</i>	glycine cleavage system
PA2942.1	-2.10	7.45E-03	P15	non-coding RNA
PA3552	-2.07	2.51E-03	<i>arnB</i>	beta-L-Ara4N-lipid A biosynthetic process
PA3621.1	-2.06	9.08E-04	<i>rsmZ</i>	regulatory RNA
PA4225	1.79	4.67E-02	<i>pchF</i>	Pyochelin biosynthesis
PA4226	1.97	1.52E-02	<i>pchE</i>	Pyochelin biosynthesis
PA4230	2.23	1.31E-02	<i>pchB</i>	Pyochelin biosynthesis
PA4231	2.01	2.55E-02	<i>pchA</i>	Pyochelin biosynthesis
PA4315	-1.58	6.23E-04	<i>mvaT</i>	transcriptional regulator MvaT, P16 subunit
PA4572	1.59	1.20E-03	<i>fkfB</i>	protein folding
PA4771	-1.63	3.79E-02	<i>lldD</i>	L-lactate dehydrogenase
PA5170	-1.88	3.85E-03	<i>arcD</i>	arginine/ornithine antiporter
PA5360	1.89	2.25E-03	<i>phoB</i>	two-component response regulator
PA5361	1.84	1.31E-02	<i>phoR</i>	two-component response regulator
PA5367	1.67	1.46E-03	<i>pstA</i>	phosphate transport
PA5368	2.09	1.75E-05	<i>pstC</i>	phosphate transport
PA5369	2.35	3.61E-05	<i>pstS</i>	phosphate transport
Nuli-1 (15min vs 0min)				
PA0280	2.56	2.4E-08	<i>cysA</i>	sulphate transport
PA0282	1.61	4.8E-03	<i>cysT</i>	sulphate transport
PA0296	-1.76	1.2E-05	<i>spul</i>	Glutamylpolyamine syntheses
PA0298	-1.57	1.4E-04	<i>spuB</i>	Glutamylpolyamine syntheses
PA0527,1	-1.98	5.8E-03	<i>rsmY</i>	Regulatory RNA

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PA0745	-1.76	4.4E-03	<i>dspI</i>	diffusible signal factor (DSF) CDA biosynthesis
PA0865	-2.20	1.3E-10	<i>hpd</i>	tyrosine catabolism
PA0866	-2.14	2.7E-04	<i>aroP2</i>	aromatic amino acid transport
PA0871	-1.75	9.6E-03	<i>phhB</i>	Phenylalanine metabolism
PA0887	-1.74	6.6E-04	<i>acsA</i>	acetyl-CoA biosynthetic process from acetate
PA1071	-1.95	1.4E-04	<i>braF</i>	branched-chain amino acid transport
PA1337	-1.88	5.8E-05	<i>ansB</i>	Glutamine metabolism
PA1338	-1.97	6.0E-05	<i>ggt</i>	Taurine and hypotaurine metabolism
PA1342	-1.63	1.3E-03	<i>aatJ</i>	Acidic amino acid transport
PA1431	-2.40	9.0E-07	<i>rsaL</i>	regulatory protein
PA1687	2.09	1.8E-04	<i>speE</i>	spermidine biosynthetic process
PA1869	1.97	2.6E-02	<i>acpI</i>	fatty acid synthesis
PA2000	-2.57	9.5E-03	<i>dhcB</i>	Valine, leucine and isoleucine degradation
PA2001	-1.53	3.3E-02	<i>atoB</i>	acetyl-CoA acetyltransferase
PA2015	-1.58	6.4E-03	<i>liuA</i>	leucine/isovalerate metabolism
PA2016	-2.48	1.9E-03	<i>liuR</i>	leucine/isovalerate metabolism
PA2356	2.37	4.3E-02	<i>msuD</i>	sulphur metabolism
PA2425	1.50	1.7E-03	<i>pvdG</i>	pyoverdine biosynthetic process
PA2444	3.07	1.8E-05	<i>glyA2</i>	Glycine, serine and threonine metabolism
PA2445	3.14	2.0E-11	<i>gcvP2</i>	glycine cleavage system
PA2446	3.00	2.8E-03	<i>gcvH2</i>	glycine cleavage system
PA2637	-1.52	2.3E-02	<i>nuoA</i>	oxidative phosphorylation
PA2736.1	-2.09	1.0E-02	tRNA-Pro	Aminoacyl-tRNA biosynthesis and tRNA charging
PA2859	1.72	8.1E-03	<i>greB</i>	transcription elongation facto
PA2942.1	-1.86	2.3E-02	P15	non-coding RNA
PA3059	1.72	4.5E-02	<i>pelF</i>	Extracellular polysaccharide biosynthesis
PA3061	2.15	8.7E-03	<i>pelD</i>	Extracellular polysaccharide biosynthesis
PA3152	-2.02	1.5E-04	<i>hisH2</i>	histidine metabolism
PA3153	-1.65	2.4E-03	<i>wzx</i>	LPS biosynthesis
PA3479	2.33	6.3E-03	<i>rhlA</i>	rhamnolipid biosynthesis
PA3621.1	-1.88	7.5E-04	<i>rsmZ</i>	Regulatory RNA
PA3885	2.17	1.1E-02	<i>tpbA</i>	protein tyrosine phosphatase

PA3935	1.75	8.6E-03	<i>tauD</i>	Taurine metabolism
PA4191	2.02	5.3E-03	isopenicillin-N synthase	isopenicillin N syntheses
PA4218	2.24	5.8E-03	<i>ampP</i>	regulation of beta-lactamase activity
PA4219	2.24	7.5E-03	<i>ampO</i>	regulation of beta-lactamase activity
PA4221	2.44	1.0E-03	<i>fptA</i>	Fe (III)-pyochelin outer membrane receptor
PA4224	2.37	1.2E-03	<i>pchF</i>	Pyochelin biosynthesis
PA4225	2.43	3.8E-04	<i>pchG</i>	Pyochelin biosynthesis
PA4226	2.45	7.5E-04	<i>pchE</i>	Pyochelin biosynthesis
PA4230	3.06	1.2E-04	<i>pchB</i>	Pyochelin biosynthesis
PA4315	-2.15	4.5E-07	<i>mvaT</i>	transcriptional regulator MvaT, P16 subunit
PA4550	-1.71	5.3E-03	<i>fimU</i>	type IV pili assembly
PA4552	-1.76	1.7E-03	<i>pilW</i>	type IV pili assembly
PA4572	2.18	3.8E-05	<i>fkiB</i>	Protein folding
PA4690a	1.72	1.8E-02	5S ribosomal RNA	
PA4770	-1.82	5.2E-04	<i>lldP</i>	Lactate oxidation
PA4771	-2.01	6.8E-03	<i>lldD</i>	Lactate oxidation
PA4773	2.11	3.5E-03	<i>speD2</i>	spermidine biosynthetic process
PA4774	2.17	1.3E-03	<i>speE2</i>	spermidine biosynthetic process
PA4777	1.84	2.7E-02	<i>pmrB</i>	two-component regulator system
PA4932	1.56	1.7E-06	<i>rplI</i>	50S ribosomal protein L9
PA5360	2.04	3.6E-03	<i>phoB</i>	two-component response regulator
PA5361	1.80	4.2E-03	<i>phoR</i>	two-component response regulator
PA5368	1.62	1.3E-04	<i>pstC</i>	phosphate transport
PA5369	2.75	1.4E-07	<i>pstS</i>	phosphate transport
PA5421	-2.03	1.0E-02	<i>fdhA</i>	Carbon metabolism

Table S4.3- *P. aeruginosa* PA01 genes that are differentially expressed in all bacterial growth conditions (LB medium, MCCM medium, and Nuli-1).

Locus tag	Gene name / Function
PA0865	<i>hpd</i> : 4-hydroxyphenylpyruvate dioxygenase
PA0625	Probable bacteriophage protein

PA0633	Hypothetical protein
PA4290	Probable chemotaxis transducer
PA0622	Probable bacteriophage protein
PA0626	Hypothetical protein
PA0638	Probable bacteriophage protein
PA0641	Probable bacteriophage protein
PA1053	Conserved hypothetical protein
PA1338	<i>ggt</i> : Glutathione hydrolase large chain
PA2015	<i>liuA</i> : Putative isovaleryl-CoA dehydrogenase
PA2204	Putative binding protein component of ABC transporter
PA2446	<i>gcvH2</i> : Glycine cleavage system H protein 1
PA3187	Probable ATP-binding component of ABC transporter
PA3235	Conserved hypothetical protein
PA3885	<i>tpbA</i> : Protein tyrosine phosphatase
PA4774	<i>speE2</i> : Polyamine aminopropyltransferase 2

Table S4.4- *P. aeruginosa* PAO1 genes that are differentially expressed only in Nuli-1 condition, being absent the genes that codifies for hypothetical proteins.

Locus tag	Gene name	Function
PA0022	<i>tsaC</i>	Threonylcarbamoyl-AMP synthase
PA0282	<i>cysT</i>	Sulphate transport system permease protein
PA0296	<i>spul</i>	Probable glutamine synthetase
PA0298	<i>spuB</i>	Probable glutamine synthetase
PA0527.1	<i>rsmY</i>	Regulatory RNA
PA0745	Dspl	diffusible signal factor (DSF) in CDA biosynthesis
PA0871	<i>phhB</i>	Pterin-4-alpha-carbinolamine dehydratase
PA1071	<i>braF</i>	High-affinity branched-chain amino acid transport ATP-binding protein
PA1431	<i>rsaL</i>	Regulatory protein RsaL
PA1869	<i>acpP2</i>	Acyl carrier protein 2
PA2000	<i>dhcB</i>	dehydrocarnitine CoA transferase, subunit B
PA2001	<i>atoB</i>	Acetyl-CoA acetyltransferase
PA2444	<i>glyA2</i>	Serine hydroxymethyltransferase 2

PA2736.1	<i>tRNA-Pro</i>	Aminoacyl-tRNA biosynthesis
PA2942.1	P15	ncRNA
PA3059	<i>pelF</i>	extracellular polysaccharide biosynthesis
PA3061	<i>pelD</i>	extracellular polysaccharide biosynthesis
PA3152	<i>hisH2</i>	Imidazole glycerol phosphate synthase subunit HisH 2
PA3280	<i>oprO</i>	Pyrophosphate-specific outer membrane porin
PA3479	<i>rhlA</i>	rhamnolipid surfactant biosynthesis
PA3621.1	<i>rsmZ</i>	Regulatory RNA
PA3824.1	<i>tRNA-Leu</i>	Aminoacyl-tRNA biosynthesis
PA3935	<i>tauD</i>	Taurine dioxygenase
PA3936	probable permease of ABC taurine transporter	
PA3937	<i>tauB</i>	Taurine import ATP-binding protein
PA4191	isopenicillin-N synthase	
PA4219	<i>ampP</i>	regulation of beta-lactamase activity
PA4230	<i>pchB</i>	Pyochelin biosynthesis
PA4231	<i>pchA</i>	Pyochelin biosynthesis
PA4315	<i>mvaT</i>	Transcriptional regulator MvaT, P16 subunit
PA4541.3	tRNA-Asn	
PA4552	<i>pilW</i>	Type 4 fimbrial biogenesis protein
PA4690a	Paraquat-inducible protein A	
PA4770	<i>ildP</i>	Lactate transport
PA4771	<i>ildD</i>	Lactate conversion to pyruvate
PA4772	probable ferredoxin	
PA4777	<i>pmrB</i>	PmrB: two-component regulator system signal sensor kinase
PA5360	<i>phoB</i>	Phosphate regulon transcriptional regulatory protein
PA5361	<i>phoR</i>	Phosphate regulon sensor protein PhoR
PA5367	<i>pstA</i>	Phosphate transport system permease protein
PA5368	<i>pstC</i>	Membrane protein component of ABC phosphate transporter
PA5421	<i>fdhA</i>	Glutathione-independent formaldehyde dehydrogenase

**CHAPTER V: DELETION OF NON-ESSENTIAL GENES
FROM LUZ19 GENOME AND *IN VITRO* EVALUATION**

Abstract

In this Chapter, a rapid and relatively low-cost method was applied to obtain phages with smaller genomes that lack non-essential genes and are adapted to infect their hosts in specific laboratory conditions. For this purpose, a well characterized *P. aeruginosa* infecting phage (LUZ19) was exposed to mutagenic agent HA and ten cycles of heat treatment in the presence of a chelating agent (EDTA). The surviving phages from each cycle of heat treatment were amplified in bacterial cultures grown in two different media (LB media and MCCM media). The analysis of the genomes of the surviving phages (LUZ19:K01, LUZ19:K02, LUZ19:K03, LUZ19:K04, LUZ19:K05) demonstrated that these contain large genomic deletions (up to 3kb) in their early genes (unknown genes, with exception of gp4). These mutants revealed the same latent period compared to the wildtype phage, although the burst size changed. All mutants showed reduced lytic spectra, which might be associated with a mutation found in the tail fiber protein. Apart from K03, all mutants had similar efficiency in killing exponentially growing bacteria. Interestingly, mutant K02 showed the same potential in reducing 24h-old biofilms and stationary-phase bacterial cultures compared with the parental phage. Nevertheless, the K02 mutant appeared to be less competitive than the LUZ19 phage in long term.

Overall, this study establishes a simple methodology to obtain phages with deleted genes that are naturally selected by heat stress, as an alternative to the existing phage genome engineering platforms. This pipeline enables the straightforward generation of mutants of non-essential genes. In turn, these mutants can provide clues towards understanding these unknown genes and potentially drive the discovery of novel phage sequences and phage core infection mechanisms at the molecular level.

5.1 Introduction

Phages are widely spread in nature and are remarkably diverse. Their genomes are densely coded, yet contain many unverified proteins and sequences of unknown function [1]. During the early stage of phage infection, these unknown coding sequences (CDS) are mainly transcribed and translated, and their products are generally known to mediate host machinery takeover, as well as encode small proteins that are toxic to the bacterial cell, yet the vast majority of specific mechanisms remain undiscovered [2,3]. Elucidating the role of CDS has potentiated the discovery of novel mechanisms of bacterial machinery hijacking [4,5], phage genome transcription regulation [6], phage-encoded inhibitors of bacterial DNA replication [7], differentiation between phage essential and non-essential genes [8], the discovery of anti-CRISPR/Cas proteins [9], phage-encoded R-M inhibitors [10,11], QS modulators [12], modulation of host global regulators [13], phage peptides that interfere on biofilm and motility [14] and bacterial metabolism [15]. These findings are usually attained by individual screening of phage proteins that induce interesting changes in bacteria (e.g., cellular toxicity) or using libraries of phage genes, followed by systematic study of its interactions, characterization and cross-validation [3].

The scientific community has pointed out that phage has essential and non-essential genes used for phage replication [8,16,17]. These unidentified genes can be edited/deleted from the phage genome, since it is considered to be safer to remove genes that potentially encode undesired functions, including toxins, integration factors, virulence, and resistant-associated factors [18]. As such, editing a phage gene enables the determination of its essential nature or function according to the obtained phenotype or lack of functionality, such as it was done by Pires and collaborators, that recently obtained a synthetic *Pseudomonas* phage with a reduced genome (deleted $\approx$ 48% of unknown phage genes), without reducing its antimicrobial properties [19]. The main limitations of phage genome-engineering include the difficulties to assemble large phage genomes, low transformation efficiencies, labour-intensive screening of recombinant phages, low efficient, laborious and complex protocols, and lack of protocols for many bacterial hosts [20].

The development of phages with improved properties precedes the age of synthetic biology where historically natural evolution was harnessed (e.g., “The Appelmans protocol”) to adapt phages to certain conditions, described as “phage training” [21–25]. For instance, this has been done to increase CF *P. aeruginosa* bacterial isolates clearance and lowering phage resistance [26]. Therefore, by exposing phages to harsh conditions, such as ultraviolet radiation [27], mutagenic chemicals (e.g., HA [28]), or

high temperatures [29], it is possible to select evolved and more resilient phages, that usually lack non-essential genes in their genome.

In this chapter, *Pseudomonas* LUZ19 phage was subjected to random mutagenesis, and heat treatment combined with a chelating agent to select for phage mutants with smaller genomes. LUZ19 phage is a well-characterized phage [30–34] that contains twenty-seven CDS annotated as hypothetical proteins, from which some encode non-essential proteins for phage replication. Thus, obtention of LUZ19 phage mutants that lack unknown genes might be considered safer for phage therapy context, however its efficacy should be evaluated. Primarily, these mutants can be studied for functionality analysis by making phenotypic observations as a traditional ‘reverse genetics’ approach. This study provides a proof of concept that minimal phage genomes can be achieved by using straightforward techniques (like heat treatments) to screen for naturally occurring mutants that are more resilient to environmental factors such as temperature.

5.2 Methods and Materials

5.2.1 Bacterial strains, phage propagation, and growth conditions

The reference strain *P. aeruginosa* PAO1 (DSM22644) from The German Collection of Microorganisms and Cell Cultures was used in all experiments and was grown at 37°C on Lysogeny Broth (LB) or LB agar (LB with 1.2% (w/v) of agar), or in Dulbecco’s Modified Eagle’s Medium: Nutrient Mixture F-12 (DMEM:F12) supplemented with 2% (v/v) of Ultrosor-G Serum Substitute Medium. This medium supports the growth of human airway epithelial cells and was chosen to mimic human bronchial physiological conditions, and is referred as MCCM [35]. *Pseudomonas phage* LUZ19 [31] was propagated on *P. aeruginosa* PAO1 cells by standard soft agar overlay, followed by PEG8000 precipitation and stored in phage buffer pH 7.5 (10mM Tris-HCl, 10mM MgSO₄, 150mM NaCl) at 4°C [36]. Phage titration was performed using the double-agar layer [37].

5.2.2 Random mutagenesis with HA

Phage were exposed to a concentration of 0.4 M of mutagenic agent HA, which permeates into phage capsid and modifies the C4 of the cytosine pyrimidine ring by addition of a hydroxy amino group, generating G: C transition to A: T, according to [38]. Briefly, an aliquot of 200µL of LUZ19 phage lysate (10⁹ to 10¹⁰PFU/mL) was mixed with 400µL of 2M HA (pH=6), 400µL phosphate–EDTA Buffer, 200µL of 100mM MgSO₄, and 400µL double-distilled water. The reaction was incubated at 37°C for 24h. Next,

the phage titer was measured by plaque overlay technique to evaluate mutagenic action in phage particle viability. Surviving phage particles were purified from the mutagenic mixture by dialyzing three times on an Amicon Ultra-0.5 Centrifugal Filter Unit 10K (Millipore) against SM buffer. After this step, the phage titer was also determined.

5.2.3 Phage mutants' selection through lysates exposure to combined heat and chelating agent treatment

Phage lysate (1mL) obtained after purification from mutagenic solution was added EDTA to a final concentration of 0.01M. This mixture was incubated at different temperatures (55, 60, 65°C) and periods (15, 30, and 60min). After each phage treatment the titer was quantified. The temperature of 65°C for 20min was selected as the best treatment to reduce phage titer, which ranged between 3-4 Log (PFU/mL). Following heat treatment, surviving phages were propagated on mid-exponential bacterial cultures ($OD_{600\text{ nm}}=0.5$) grown in either LB and MCCM medium until cultures were cleared. Lysates were centrifuged (9,000×g, 4°C, 15min) and filtered (0.22µM) to remove cellular debris. In total, ten cycles of heat treatment with EDTA followed by phage propagation in liquid media was made, being quantified always the phage titer after heat treatment and after propagation.

5.2.4 Phage mutant isolation

Surviving phages (resulting from ten cycles of heat treatment/EDTA and consecutive enrichment in LB or MCCM medium) were serially diluted in SM buffer. Each dilution (100µL) was plated by the double-agar layer method. Several clear plaques with a different morphology from wildtype LUZ19 phage were picked, and forty rounds of propagation were made until selecting phage plaques with stable morphology. Phage plaques from newly isolated phages were selected and resuspended in 100µL of SM buffer to check for deletions in the phage genome. Mutant phages with deletions were propagated and PEG-purified as above mentioned.

5.2.5 Phage plaque PCR to screen for genome deletions

PCRs were made for each picked putative phage-mutant plaque and wildtype LUZ19 phage using the DreamTaq Green PCR Master Mix (2X) (Thermo Scientific) and primers that target LUZ19 early genes, gp1-gp10_Fw (5'-tgatggccactcagcaaaag-3') and gp1-gp10_Rv (5'-tatggtatggctagctcgca-3') respectively. The amplification procedure was followed according to the manufacturer's instructions, with a few adaptations: extension time was 4min, and 50°C was used as melting temperature. PCR products were

analysed by 0.5% agarose gel electrophoresis, with an expected PCR product size of around 4,383bp for LUZ19 phage without deletions.

5.2.6 Phage DNA extraction and sequencing

The isolation of phage DNA was done using the Quick-DNA Viral Kit (Zymo Research) following the manufacturer's instructions. Phage DNA libraries were constructed using the Illumina Nextera XT library preparation kit (Illumina) and sequenced on aminiSeq sequencer (Illumina) using 150bp paired-end sequencing reads. The raw sequencing data was trimmed using BBDuk trimmer package from Geneious R11 to eliminate sequencing adaptors and low quality/length reads. Then, reads were mapped to the LUZ19 genome (access number NC_010326) using Geneious mapper. An alignment was performed between consensus sequences from each phage mutant and LUZ19 genome to track deletions and polymorphisms.

5.2.7 Phage infection of exponential-bacterial cultures

Overnight *P. aeruginosa* PAO1 cultures grown at 37°C with agitation (120rpm) were diluted 1:100 in a final volume of 20mL of LB medium. Cultures were incubated at 37°C and 120rpm until reaching an OD_{600nm} of 0.5. Then, mutant phages and LUZ19 phage were individually added at final MOI=1 and OD was measured at 30min intervals until 2h post-infection.

5.2.8 One-step growth curves (OSGC)

Phage infection parameters were determined accordingly to [19]. Briefly, 10 mL of an early-exponential phase culture of *P. aeruginosa* PAO1 grown in LB medium (OD₆₀₀ of 0.3, i.e., 3×10^8 CFU/mL) was centrifuged (7,000×g, 4 °C, 5min) and resuspended in fresh LB medium. The culture was infected with mutant phages at an MOI of 0.01, and phage adsorption was allowed for 5min at 37 °C under agitation. Non-adsorbed phages were removed by centrifugation of infected cultures at 7,000×g, 4°C, 5min. The cell pellet were resuspended in 10 mL of fresh medium. At this point, a sample was taken immediately (t=0min) and the culture was incubated at 37°C with agitation, being taken samples at each 5min period. Each sample was taken in duplicate and serially diluted to evaluate phage particle concentration by double-agar layer.

5.2.9 Phage host range

Phage infectivity of LUZ19 and phage mutants were assessed towards 28 *P. aeruginosa* clinical strains. Specifically, bacterial lawns were prepared for each bacterial isolate by spotting 5µL of phage solution on each bacterial lawn and allowed to dry. Next, plates were incubated overnight at 37°C. Susceptible strains were challenged with serial dilutions of phage suspensions to determine the efficiency of plating (EOP).

5.2.10 Phage efficacy against 24 h-old biofilms

P. aeruginosa PAO1 overnight cultures grown in LB medium at 37°C and 120rpm were diluted 1:100 in fresh LB and then 200µL of suspension was transferred to each well of 96-well polystyrene microtiter plates, which was incubated 24h at 37°C and 120rpm. Thereafter, planktonic bacteria were removed, and each well was washed twice with 200µL of fresh LB. Afterward, 200µL of phage in LB medium was added to each well (LUZ19, KO2, and SM buffer as control) at a final MOI=0.1. The plates were incubated for 2, 4, 6, 8, 12, and 24h at 37°C with 120rpm. At each time point, the supernatant was removed, and wells were washed twice saline [0.9% (w/v) NaCl]. Finally, biofilms were scraped in 200µL of LB medium, and samples were collected and diluted in 5 mM of ferrous ammonium sulphate (FAS), to prevent further infection of bacteria by phage. Dilutions were used to quantify colony forming units (CFU) by running 10µL of each dilution over an LB agar plate to count single colonies. Plates were incubated overnight at 37°C. At each time point, triplicate samples were collected, and three independent assays were performed in total.

5.2.11 Competition assay

A mixture of mutant KO2 and wildtype LUZ19 phages (1:1) was used to infect a *P. aeruginosa* PAO1 culture at an OD₆₀₀=0.3, grown in LB, with 0.01 MOI. The suspension was incubated for 1h at 37°C with agitation. At the end of this period (defined as cycle 1), the suspension was centrifuged at 9,000×g, 4°C, 15min. The supernatant was 0.2µM filtered. A 1:1,000 dilution of this phage-mixture lysate was transferred from one cycle to the next, resulting in increasing effect over time for six cycles in total. To quantify the relative abundance of each phage genome, phage DNA was extracted at cycles 0, 3, and 6, and sequenced as mentioned above. Trimmed reads obtained for each time-sequenced sample were mapped to the LUZ19 genome. Using the average coverage, the region that comprises the deletion zone in the phage mutant was determined. Indeed, the coverage for phage mutant was obtained by subtracting it from the total average for wildtype LUZ19 phage. With the known average coverage for both wildtype

and mutant phages, it was calculated a ratio between each phage and a percentage, and plotted for subsequent cycles to represent how both phages' abundance varied.

5.3 Results

5.3.1 Optimization of heat treatment procedure aiming at the production of heat-resistant phages

Acquisition of phage mutants with large genomic deletions was achieved through challenging phage LUZ19 lysates to heat treatment. For that, LUZ19 phage was incubated with HA to induce random point mutations in the phage genome, followed by a selection of surviving mutants that are also able to resist to several cycles of heat treatment/EDTA exposure. After exposure of phage lysate to HA for 24h, a significant reduction in phage titer was observed (3.55Log reduction of PFU/mL), indicating that mutations caused by HA lead to a high extent of phage particle loss. After removal of HA, phage lysate was heat treated under EDTA presence to select for phages that have mutations in phage genes. In a first step, the wildtype phage LUZ19 was tested against several temperatures and incubation times to understand its resistance to the mentioned treatment. It was observed that the phage was quite resistant to temperatures of 55 and 60°C, independently of the incubation period (up to 1h) (Table 5.1). By contrast, a significant drop in phage titer was observed at 65°C, resulting in a 4.41 Log reduction after 1h. Taking advantage of the greater reduction in phage titer after one cycle of treatment at 65°C up to 30min, and considering that this can potentially select for heat-resistant phages with smaller genomes, the phage lysates were exposed to an intermediate treatment in order to obtain a $\approx 2\text{-}3\text{Log}$ reduction of phage particles. As such, 20min at 65°C was selected as the best treatment, with a 3.44Log reduction after one cycle of treatment, and this was applied to the phage mixture that resulted after random mutagenesis treatment. Surviving phages were propagated in LB medium and in MCCM medium to enrich for phages that potentially might have different fitness according to medium conditions used to grow bacteria.

During the selection of putative mutant phages, using heat treatment followed by phage propagation, oscillations in the phage titer were observed over the procedure, with a slight decrease in Log (PFU/mL) reduction after heat treatments. The stabilization of the phage concentration loss (Table 5.2) induced by heat treatment over the number of cycles was interpreted as a potential increase in heat-tolerant phages within the viral population.

Table 5.1- Titer reduction of wildtype LUZ19 phage lysates with 0.1M of EDTA when heat treated to different temperatures and periods.

Thermic treatment	Log reduction
55°C_15min	0.54
55°C_30min	0.57
55°C_60min	0.71
60°C_15min	1.17
60°C_30min	1.41
60°C_60min	1.93
65°C_15min	2.65
65°C_30min	4.06
65°C_60min	4.41

Table 5.2- Log reduction of phage titer lysates obtained after production in bacterial cultures grown either in LB or MCCM media after each cycle of heat treatment.

Cycle	LB medium	MCCM medium
1	2.66	
2	2.06	2.62
3	3.13	6.68
4	2.744	4.88
5	3.92	5.79
6	2.58	3.25
7	2.03	3.09
8	1.8	3.33
9	1.48	2.89
10	1.5	1.92

5.3.2 Screening of phage mutants with large deletions

The final phage mixtures resulting from mutagenic agent/heat-treatment were plated on bacterial lawns to observe phage plaque morphology and compare to the wildtype phage. Phage LUZ19 generates large plaques between 5 to 10mm, although lysates from heat treatments showed a high variability of

phage-plaque sizes (Figure 5.1). The different-sized phage plaques were picked and propagated in bacterial lawns until a stable plaque morphology was observed (≈ 40 passages). Then, different isolated phage plaques were analysed by PCR targeting genes between gp0.1 to gp11 (which are mainly annotated as hypothetical genes) to screen for phages that potentially lost non-essential genes. PCR products from picked “hypothetical mutant phages” demonstrated smaller sizes compared with PCR products obtained for wildtype phage ($\approx 4,383$ bp), indicating a deletion of around 2-3kb (Figure S5.1). Interestingly, the mutants originated from propagation in the MCCM medium weren’t even able to give PCR products with the used primers set targeting early genes, which indicated even larger deletions that start before gp0.1, or end after gp10.

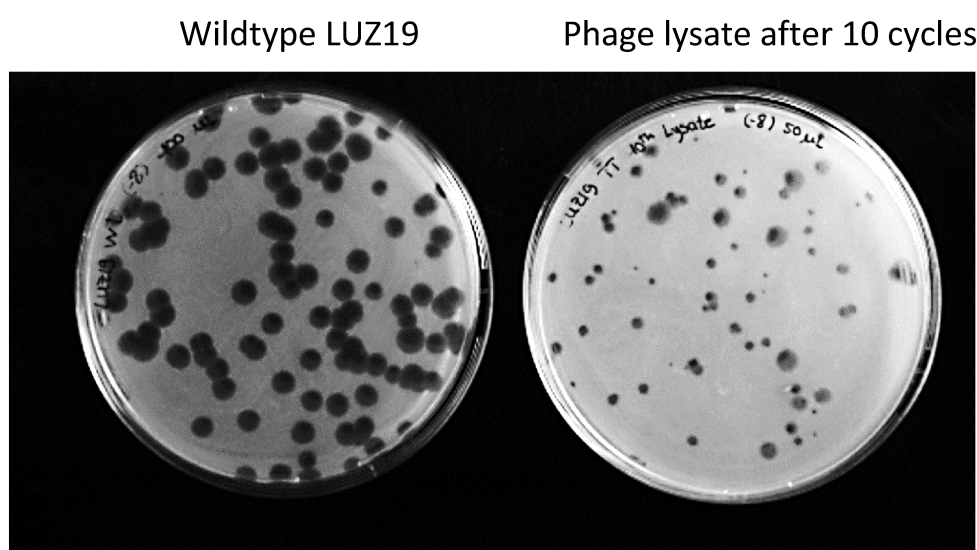


Figure 5.1- *P. aeruginosa* PAO1 bacterial lawns with diluted lysate from wildtype LUZ19 phage and with lysate obtained after 10 consecutive cycles of heat treatment in the presence of 0.1M EDTA and enrichment in bacterial cultures grown in LB medium.

5.3.3 Phage deletion mutants’ validation

Considering that almost all picked phages were mutants with possible deletions in the region of the early genes, five of these phages (LUZ19:KO1, LUZ19:KO2, LUZ19:KO3, LUZ19:KO4, LUZ19:KO5) were confirmed by whole genome sequencing (Figure S5.2, Table S5.1). Sequencing of mutant phages was analysed by mapping the reads to the LUZ19 phage genome, being visualized a considerable gap in the phage genome where no reads were mapped (Figure 5.2). Looking closer to these regions, the start and end of the deletions were tracked, which revealed deletion sizes around 2.5 to 3.2kb, and their location was very similar among the five mutants (between gp3 and gp11). Interestingly, these deletions were all flanked by homologous regions of about 7 to 8 nucleotides (Table S5.2).

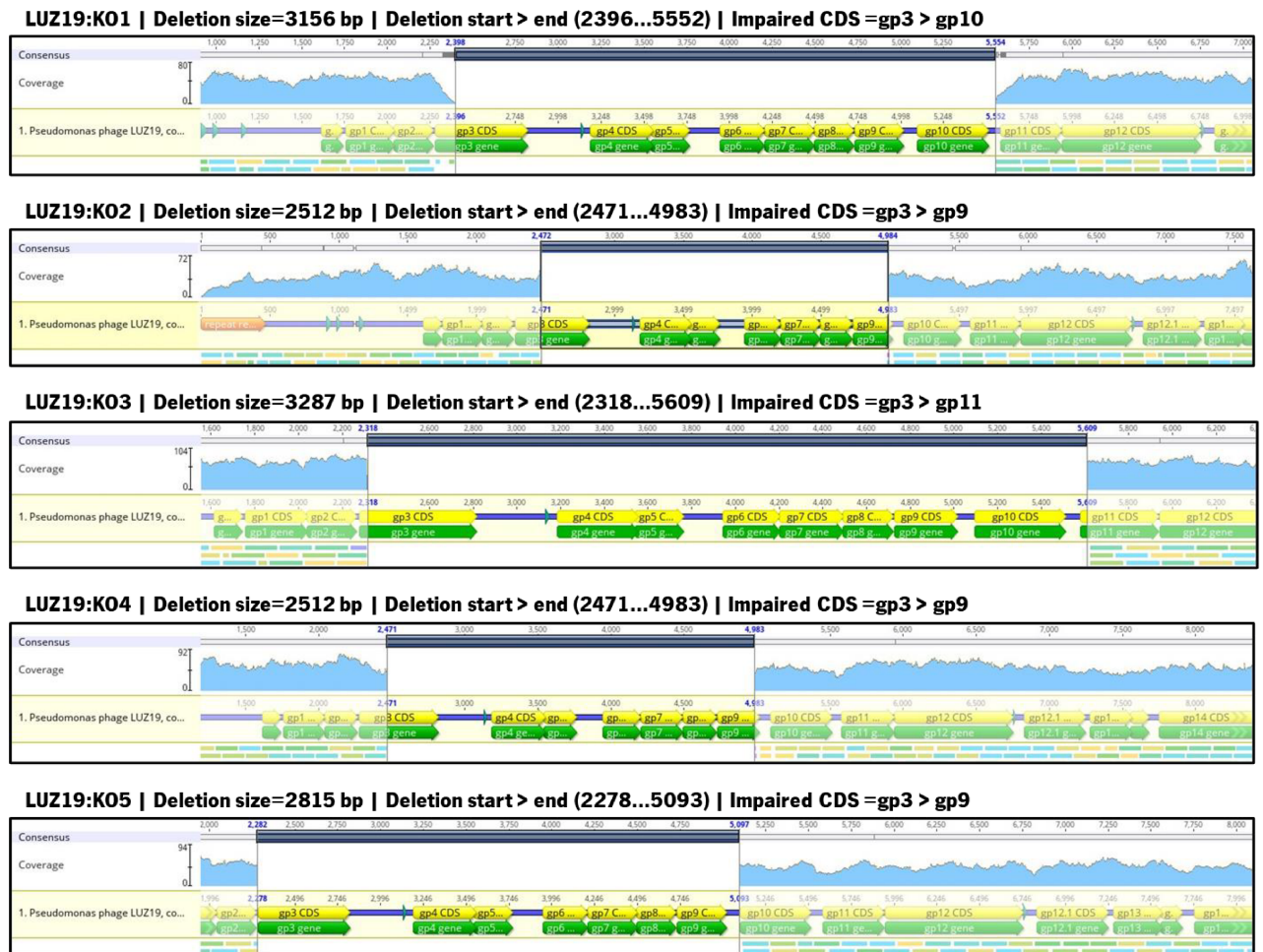


Figure 5.2- Visualization of deletion region for each phage mutant. Reads from sequencing of each phage mutant were mapped to the LUZ19 genome using Geneious software.

In addition to deletions, single nucleotide polymorphisms in phage mutants were also verified to understand if the mutagenic agent/heat treatment had also generated mutations across the entire genome. Many SNPs were observed for mutant LUZ19:K01 in particular (Table S5.3). In addition, some SNPs were found to be common among the phage mutants, providing all mutants with a specific common mutation, in a tail fiber (Table S5.4).

5.3.4 Phage mutants' characterization

Infection parameters of phage mutants during *P. aeruginosa* PAO1 infection were evaluated by OSGC where bacterial suspensions were infected with a 0.01 MOI. Compared with wildtype phage (data kindly provided by Dr. Diana P. Pires), all phage mutants revealed a similar latent period and comparable to LUZ19 phage. However, considering the burst size, all mutants showed differences, especially LUZ19:K01 and LUZ19:K03 (with smaller burst size), and LUZ19:K02 with a higher burst size (Figure

5.3). Statistically significant difference was only found between LUZ19:K02 and LUZ19:K03 (p-value=0.0168).

In addition, the host range and EOP were tested to compare the phage mutant's infectivity with the wildtype phage against a set of *P. aeruginosa* clinical strains. In general, mutant phages revealed a reduced ability to infect the tested strains in comparison to phage LUZ19 (Table S5.5 and Table S5.6), which may indicate that or the deletion of early genes is involved in this inability to infect other strains, or this limitation is imposed by one of the emerged mutations spread over phage genomes.

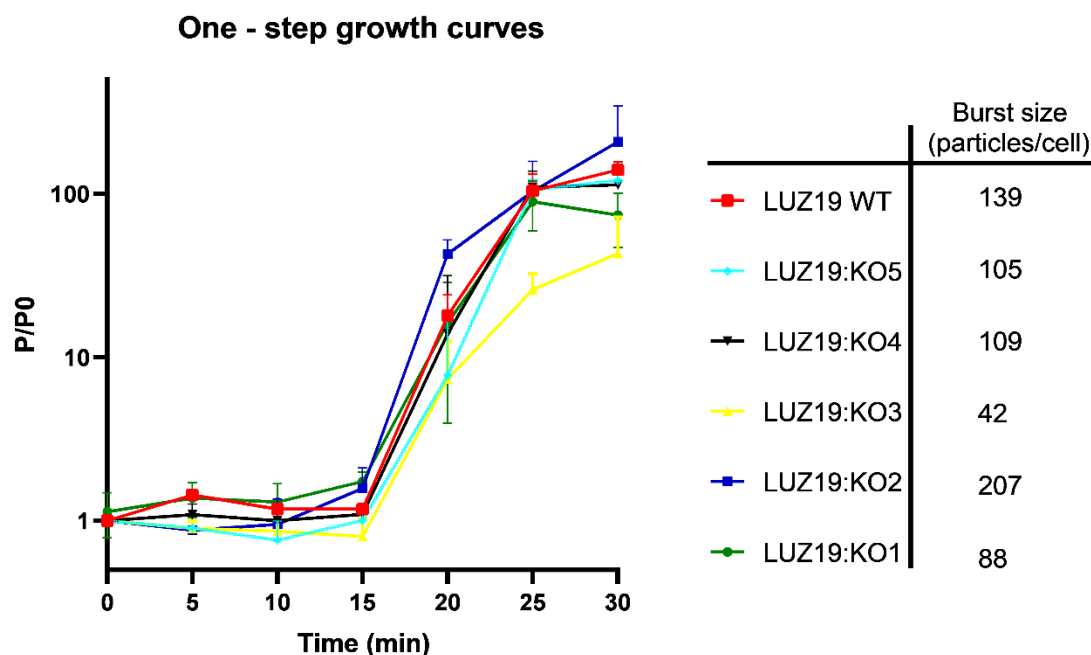


Figure 5.3- OSGC for each phage mutants (LUZ19:K01, LUZ19:K02, LUZ19:K03, LUZ19:K04, and LUZ19:K05) and wildtype LUZ19 phage. Phage infection parameters were determined through infection of bacterial cultures with a 0.01 MOI.

Planktonic cultures of *P. aeruginosa* PAO1 bacteria at exponential phase were also challenged with mutant and wildtype LUZ19 phages to compare their ability to infect and reduce bacteria. With exception of mutant LUZ19:K04, all phages were able to quickly reduce the OD of bacterial cultures similarly to wildtype phage, while in the control (SM buffer) bacteria continues to grow (Figure 5.4).

Among all phage mutants, phage LUZ19:K02 was selected to perform deeper efficiency studies because it showed a higher burst size. Therefore, 24h-old biofilms were challenged by both phage LUZ19:K02 and phage LUZ19, with a final MOI of 0.1. Both phages were able to significantly reduce *P. aeruginosa* biofilms comparing with control at 2h (p-value<0.05), 4h (p-value<0.05), 6h (p-value<0.01),

12h (p-value<0.001), and 24h (p-value<0.001) (Figure 5.5). However, no statistical difference was observed between both phages.

To better understand the fitness of the LUZ19:K02 phage mutant, a competition assay between two phages (LUZ19:K02 vs LUZ19) was performed in *P. aeruginosa* PA01 exponential cultures.

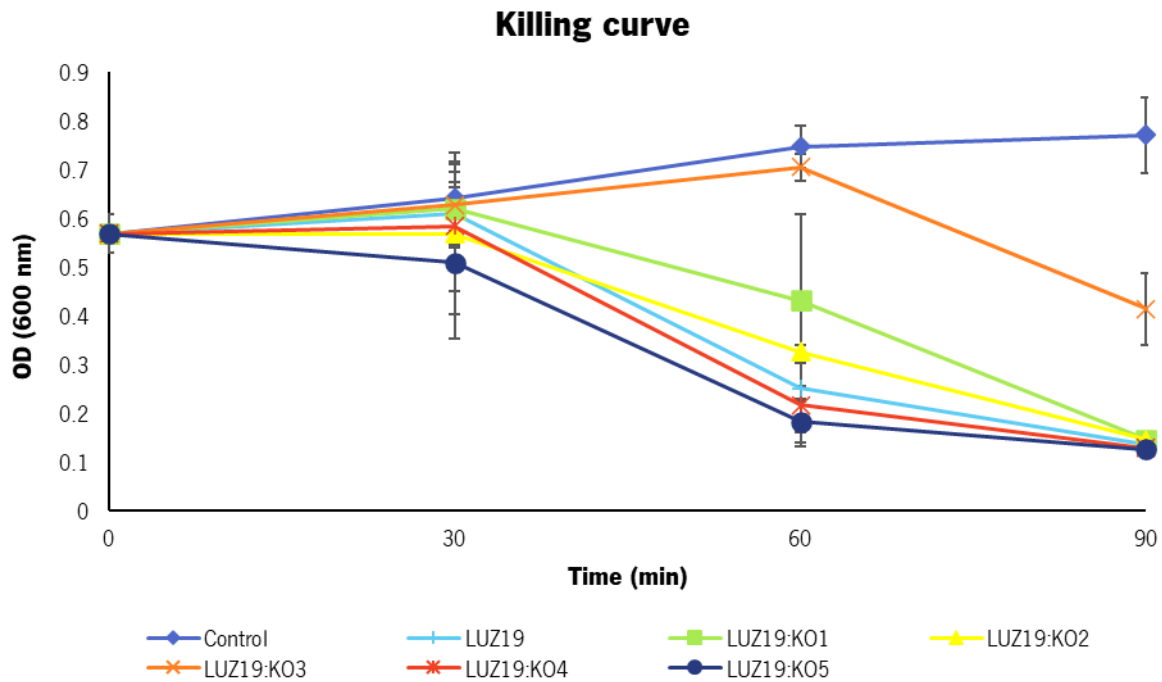


Figure 5.4- Efficiency of LUZ19, LUZ19:K01, LUZ19:K02, LUZ19:K03, LUZ19:K04, and LUZ19:K05 mutants on reducing optical density (at 600nm) of *P. aeruginosa* PA01 exponential cultures (≈ 0.55) when infected at a final MOI of 1. In the control, SM buffer was added. At each time point, OD was measured twice, and error bars represent standard deviation average values from two independent assays.

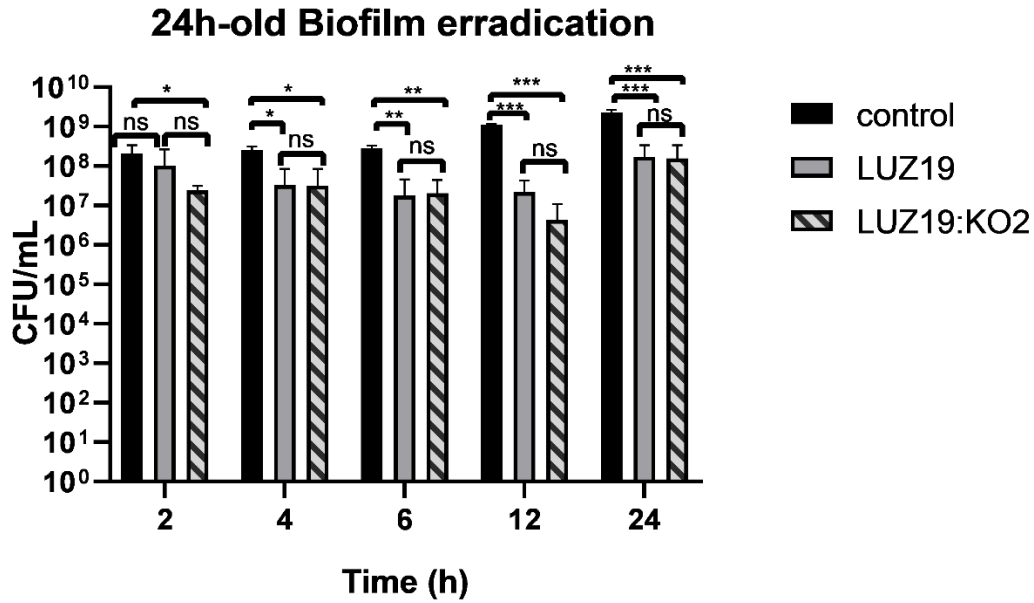


Figure 5.5- Surviving biofilm bacterial cells from a 24h- old *P. aeruginosa* PAO1 biofilm after being exposed to phage LUZ19 and mutant K02 at 0.1 MOI, after different time points. At each time point triplicates were made, and error bars represent standard deviations from three independent biological assays (ns for no significant difference, * for p-value<0.05, ** for p-value<0.01, *** for p-value<0.001).

5.3.5 Competition assays to assess phage fitness

P. aeruginosa PAO1 bacterial cultures were infected with a mixture of wildtype LUZ19 and LUZ19:K02 (1:1) for 1h at 37°C with agitation (one cycle). At the end of a cycle, a 1:1000 dilution of phage suspension was added to infect a newly grown bacterial culture. This was repeated until six cycles were completed. Sequencing of samples collected at the end of each cycle enabled the tracking of phage population evolution in terms of the relative quantity of each phage. The average of reads mapped to LUZ19 in the region where K02 has the deletion of early genes, allowed the estimation of both phages' abundancy for cycles 0, 3, and 6. Results showed that the initial amount of each phage was not exactly 50% probably due to pipetting errors (Figure 5.6). Even though, it is clear that over the infection cycles, wildtype phage dominates the population, being its relative abundance already 85.7% after three competition cycles, which indicates that for longer periods, the wildtype phage is better equipped to survive and has advantageous traits that contribute to its fitness, compared to the K02 phage mutant. A last test was performed just to understand both phages' ability to infect bacteria at different temperatures

and both phages are equally able to generate isolated phage plaques in bacterial lawns at RT, 30°C, 37°C, and 42°C (Table S5.7).

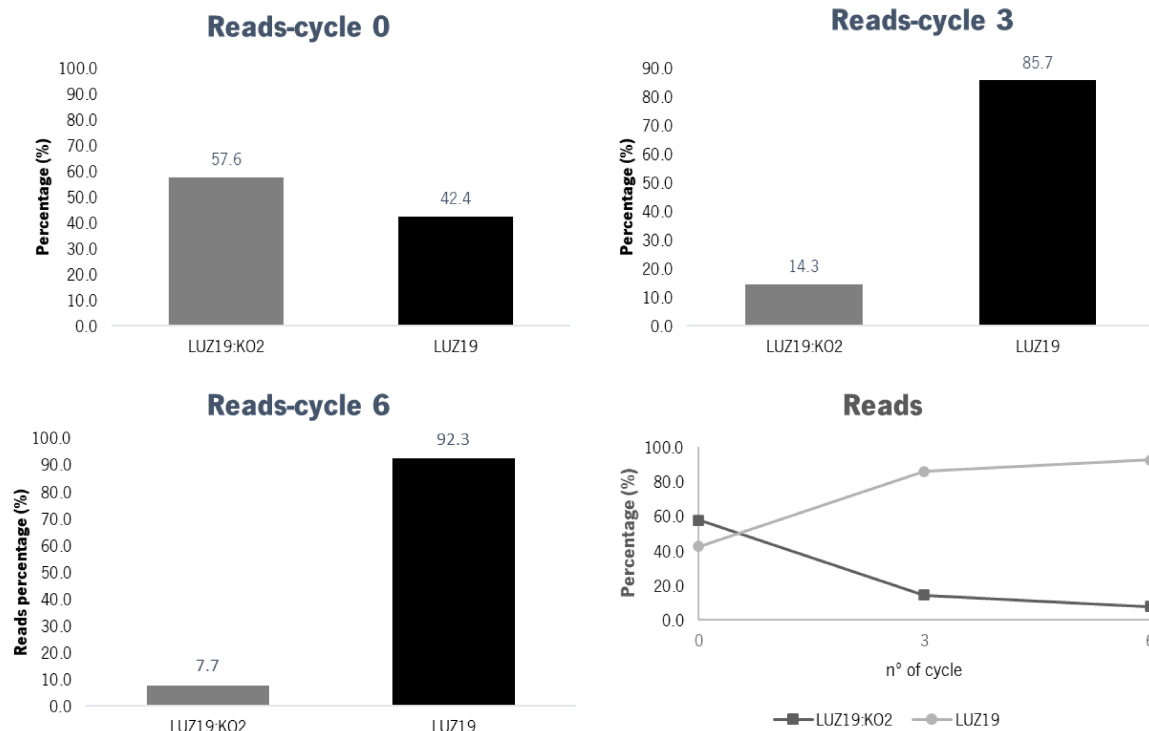


Figure 5.6- Overview of the relative abundance of LUZ19 phage and K02 phage during six cycles of competition assays, which begin with the same amount of each phage to infect *P. aeruginosa* PA01 exponential-phase cultures. Relative abundance of each phage was calculated according to average reads coverage obtained for the deletion region that is absent in the K02 phage, and therefore corresponds to LUZ19 average coverage, being the average coverage for the whole genome (except in the deletion region) subtracted with coverage for LUZ19 used to determine K02 abundance.

5.4 Discussion

Phages carry very densely coded genomes [39], and generally rely heavily on host machinery for their own reproduction [2]. This allows them to encode a restricted set of essential genes necessary for its viability, reproduction, and host hijacking. Nevertheless, phage genomes also possess non-essential genes [8,16,40,41] and, under permissive conditions, these non-essential genes can be abolished without impairing phages' growth [42]. For instance, T7 ligase mutant are not able to grow in ligase-deficient strains [43]. The main limitation is that the set of phage essential genes remains poorly defined because phages are very variable and contain many genes of unknown function [1].

5.4.1 Selection of phage mutants with large deletions through combination of HA, heat treatment and EDTA

As an alternative to transcriptomic studies, which provide great insights for screening of genes function, this study used the direct manipulation of phages by an easy and low-cost method to obtain phages with a reduced genome, which can be used to assess phage genes' function/essentiality based on its phenotypic changes comparatively to wildtype phages. This technique can also be used to identify genes that are not essential for phage replication. In total, five phage mutants of *Pseudomonas* LUZ19 were obtained. These mutants contain large deletions in their genomes, particularly affecting the early genes that hypothetically would be important for the takeover of host machinery. These mutants were generated by a combination of random mutagenesis and selection of phages resistant to high temperatures under the presence of EDTA. Phages with reduced genomes are thought to occur naturally in the phage population and have been noticed at the very beginning of phage biology research [29,42,44,45]. Many experiments have reported that the exposure of phage lysates to high temperatures results in the selection of phage variants that are heat-resistant and frequently contain genomic deletions compared with parental phage [29,42,44–48]. The presence of chelating agents such as EDTA appear to potentiate this effect [28,49]. So far, only a limited number of studies have attempted to explain the molecular process behind the selection of smaller phages through heat treatments: phage DNA is packed in the capsid under high pressure (bending stress of DNA polymer, repulsive electrostatic and hydration forces between adjacent DNA helices create an internal pressure of tens of atmospheres within the viral capsid) [48,49]; an increase in this internal pressure can lead to premature injection of packaged phage DNA, which ultimately results in viral particle inactivation [49]; high temperatures destabilize the internal pressure of packed DNA and the capsid stability, leading to DNA loss from the capsid and consequent particle inactivation [48,49].

Interesting, less dense DNA is under diminished internal pressure, resisting better to temperature-induced pressure, which means that virions with smaller genomes are more resistant to heat treatments compared to wildtype phages with full genome length [29,42,44,46,47,49,50], and heat released DNA increases with DNA length [51]. Therefore, it was used this *a priori* knowledge in a combination of using a mutagenic agent (HA) to increase the chances of LUZ19 mutation [38,47,52,53] and loss of non-essential genes as proposed by a few studies [38,42,47].

5.4.2 Genetic characteristics of phage mutants

Our results demonstrate that these temperature-resistant phages with large deletions (up to 3kb) are possible to obtain in a quick procedure (with ten cycles). During this procedure selection of thermostable phages is accompanied with a dramatic reduction of phage titer (99.9%) after each exposure of phage lysates to high temperatures, which is consistent with previous observations [46]. Finally, a plateau with a less pronounced reduction was observed, also consistent with previous observations [47]. Interestingly, these deletions were also found to occur between repeat regions that shared 7-8 nucleotides of homology. This is consistent with previous studies, supporting the hypothesis that these deletions occur by recombination potentiated by these homologous regions [46], and probably these regions might be recombination hotspots within the phage genome. In addition to genomic deletions, the applied protocol generated a substantial increase in mutations that potentially leads to SNPs. SNPs accumulation was also observed during T7 phage exposure to heat treatments [46]. To identify decreases or increases in phage efficacy, basic microbiological assays were performed, including host range, OSGC, inhibition of biofilms, and phage competition assays.

5.4.3 Phage mutants exhibit a narrower host range

A drastic decrease in the phages' ability to infect *P. aeruginosa* clinical strains was observed, which might be a result of a common SNP found in the five mutants in gp40 that encode the phage tail fiber. However, it is also possible that the loss of genes impairs the phage's ability to counteract bacterial defence mechanisms present in the clinical *P. aeruginosa* isolates. Curiously, mutations that arise during the chemically accelerated viral evolution (CAVE) protocol did not result in changes in phage infectivity, including in mutants host range [47]. Here, in addition to host range modifications, it was also observed modifications in mutants' burst size: a burst size decrease for mutants LUZ19:K01, LUZ19:K03, LUZ19:K04, and LUZ19:K05; whereas an increase was observed for mutant LUZ19:K02. This phenomenon was also observed for phage PE3, which is genetically closer to phage LUZ19. A deletion of different combinations of early genes led to mutants with differences in infection parameters (or in the latent period, or burst size, or host range) [19]. Curiously, a common mutation among PE3 mutants were also found in a tail fiber coding gene (gp47), not being known its cause yet, although authors suggest that was probably caused by PCR-associated errors. Differences in phage infection parameters were also observed after the deletion of ORF_01 from *Salmonella* phage PVP-SE2 [54] by homologous recombination, even when this gene was described to be a non-essential gene. It would be an interesting

phenomenon to study whether SNPs in phage tail fibers might be an adaptation associated with the pressure of the loss of specific phage sequences.

Considering that all mutants presented different phenotypes compared to wildtype phage, the ability to reduce *P. aeruginosa* PAO1 cultures by the different mutants was tested, which resulted in similar efficiency compared with LUZ19 phage, with exception of phage LUZ19:K04 that was slower in reducing the optical density of bacterial cultures. In general, this phage lytic performance was inferior compared to the other mutants, with a smaller burst size, reduced ability to infect clinical strains, and to kill bacterial cultures. Conversely, phage LUZ19:K02 revealed a similar antibacterial potential, but with a higher burst size, compared to wildtype LUZ19 phage and remaining mutants (K01, K04, and K05).

5.4.4 LUZ19:K02 mutant reveal similar anti-biofilm potential to the parental phage

Taking into account the higher burst size of LUZ19:K02 relative to LUZ19, its anti-biofilm properties were studied and compared with the wildtype phage. In general, LUZ19:K02 revealed similar efficiency in reducing 24h-biofilm of *P. aeruginosa*, comparing with LUZ19 phage. In addition to biofilm assays, LUZ19 phage and LUZ19:K02 are both equally able to generate phage plaques in bacterial lawns when these are incubated at different temperatures.

5.4.5 Wildtype LUZ19 prevails in competition assays over LUZ19:K02

A final test was done to understand in which sense the loss of early genes could led to a phage with better or worse fitness comparing with LUZ19 phage. For that, a competition assay similar to [55] was performed, in which bacterial cultures were infected with an equal ratio of mutant LUZ19:K02 and wildtype phage LUZ19. Over six cycles of co-infection, it was possible to observe that wildtype phage increased its abundance compared with the mutant phage, indicating that the wildtype phage has a higher fitness and prevails in the long term. This was not obvious from the reduction of biofilm, stationary, and exponential cultures results. The cause of this reduced fitness in LUZ19:K02 mutant remains unknown, which indicates that the deleted early genes might be involved in crucial roles that allow phage persistence in the long-term, or mutations over the genome impair phage propagation/survival. Indeed, recently one of these deleted genes was found to be important for LUZ19 infection, the gp4 (now denominated as Qst, QS targeting protein) that is involved in *P. aeruginosa* QS modulation [12].

Overall, mutant LUZ19:K02 revealed to be an efficient phage, similar to phage LUZ19, despite being less competitive when co-cultured with parental phage and having a reduced host range. Nevertheless, the preliminary assays were obtained under *in vitro* conditions. *In vivo* efficiency studies

would be required to guarantee the potential of using these genome-reduced phages for applications such as phage therapy. In parallel, assays that better discriminate the fitness of mutant phages need to be employed to better understand to which extent the deleted unknown genes are truly unessential, and hopefully, novel functions and mechanisms can be discovered.

5.5 Conclusions

In conclusion, it was demonstrated that phages with reduced genomes can readily be obtained, without using targeted phage engineering platforms, by taking advantage of heat selection of naturally occurring phages with genomic deletions. Furthermore, the deleted genes are not essential for phage replication under laboratory conditions, however, they might have an impact in phage antibacterial performance. The selection of genome-reduced phages can be used as an extended step to integrative omics when applied to the discovery gene's function. For instance, proteomic and transcriptomic patterns can be compared between mutant and wildtype phages, to provide further information about the role of deleted genes. Furthermore, the comparison of the lytic spectra between wildtype and mutant phages can provide clues about phage proteins involved in host recognition, or/and phage features involved in anti-bacterial defence systems, such as anti-CRISPR proteins against CRISPR/Cas systems often found in bacterial clinical isolates.

In this work, it was observed that mutants with deletions in early genes efficiently reduce bacterial cultures but exhibit different infection parameters and a narrow host range compared to the wildtype phage. In the future, it is important to determine if this limited infection is related to the early gene's deletion or with SNPs in phage tail fibers, or even both correlated through an unknown phenomenon.

Finally, it is important to mention that the methodology used in this study is an interesting and affordable tool to optimize and evolve phages toward more stable and resilient phages, which might perform better for phage therapy applications. Furthermore, this tool offers the possibility to generate phages with reduced genomes which can be advantageous for the insertion of new genes to confer phages new desirable features.

5.6 References

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5.7 Supplementary data

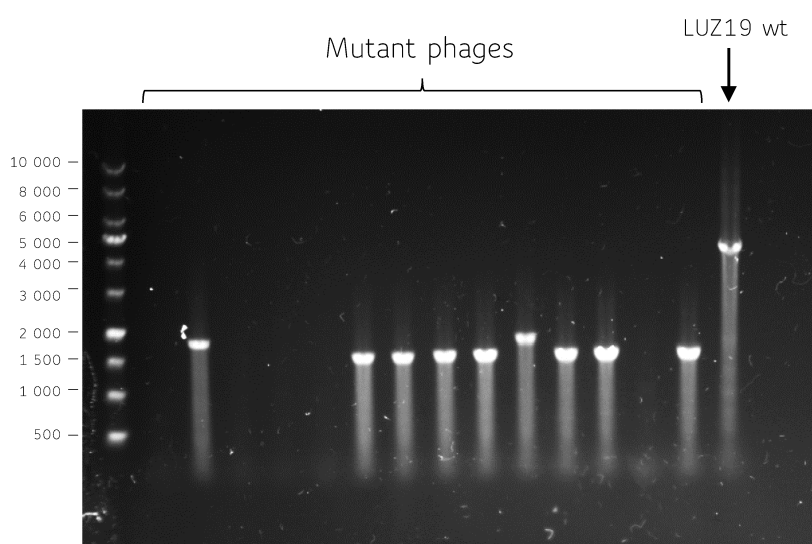


Figure S5.1- 1% Agarose gel containing PCR products of phage plaque mutants and wildtype phage LUZ19, using primers targeting gp0.1 to gp10. In the first lane it loaded to GRS

Ladder 1kb (Grisp), the following five lanes correspond to mutant phages obtained from MCCM enrichment and the remaining following lanes correspond to phages isolated from LB enrichment. In the last lane, it was loaded PCR product obtained from wildtype phage amplification with the same primers targeting gp0.1 to gp10.

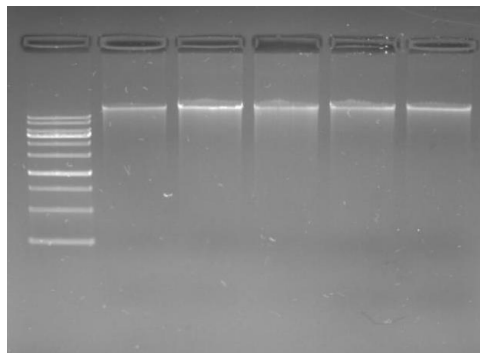


Figure S5.2- Agarose gel containing genomic DNA from mutant phages K01, K02, K03, K04, and K05. In the first lane GRS Ladder 1kb (Grisp) was loaded.

Table S5.1- Genomic DNA extraction from phage mutants using Quick-DNA Viral Kit (Zymo Research).

DNA sample	Concentration (ng/ μ L)	A260/A280	A260/A230
K01	38.2	1.94	0.66
K02	52.3	1.89	1.09
K03	56.3	1.86	1.01
K04	44.6	1.9	0.76
K05	42.3	1.91	1.38

Table S5.2- Short repeat nucleotides that are before and after each deletion site.

Phage	Repeat sequence	Size (nt)
K01	GGCACCG	7
K02	AAGGGCA	7
K03	GGCACCG	7
K04	AAGGGCA	7
K05	CATGACCA	8

Table S5.3- List of polymorphisms found in each mutant retrieved from Geneious software.

Minimum	Maximum	Amino acid change	Nucleotide change	Polymorphism type	Protein effect	Variant P-Value (approximate)
K01						
43,388	43,388		C -> T	SNP (transition)		2.5E-34
39,639	39,639		C -> T	SNP (transition)	None	1.0E-180
37,801	37,801	N -> K	C -> G	SNP (transversion)	Substitution	2.5E-202
36,465	36,465	I -> S	T -> G	SNP (transversion)	Substitution	6.3E-208
31,380	31,380		C -> T	SNP (transition)	None	4.0E-177
12,377	12,377	E -> K	G -> A	SNP (transition)	Substitution	4.9E-167
10,655	10,655	G -> D	G -> A	SNP (transition)	Substitution	2.5E-130
7,965	7,965	D -> N	G -> A	SNP (transition)	Substitution	1.6E-211
7,449	7,449		G -> A	SNP (transition)	None	1.0E-198
5,944	5,944		G -> A	SNP (transition)	None	1.0E-234
5,607	5,609	N -> R	AAC -> CGT	Substitution	Substitution	7.9E-90
5,595	5,604	PEHI -> VIAF	CCCGAACACA -> GTAATCGCAT	Substitution	Substitution	4.0E-71
5,593	5,593	N -> T	A -> C	SNP (transversion)	Substitution	2.5E-73
5,586	5,591	KR -> SK	AAGCGC -> TCCAAA	Substitution	Substitution	4.0E-72
5,584	5,584	S -> I	G -> T	SNP (transversion)	Substitution	1.0E-70
5,580	5,582	M -> A	ATG -> GCA	Substitution	Start Codon Loss	6.3E-62
5,578	5,578		C -> A	SNP (transversion)		1.0E-54
5,575	5,575		T -> C	SNP (transition)		7.9E-57
5,567	5,573		AGGAGGA -> G	Deletion		6.3E-45
5,555	5,558		TAGC -> GGTA	Substitution		1.6E-24
2,393	2,393	T -> A	A -> G	SNP (transition)	Substitution	1.6E-8
2,388	2,391		AACT -> CT	Deletion	Frame Shift	5.0E-7
2,384	2,385	I -> C	AT -> TG	Substitution	Substitution	6.3E-12
2,382	2,382	A -> G	C -> G	SNP (transversion)	Substitution	1.0E-15
2,378	2,379		GA -> TGG	Insertion	Frame Shift	2.5E-19
2,376	2,376	D -> V	A -> T	SNP (transversion)	Substitution	4.0E-18
2,371	2,372		AC -> GGA	Insertion	Frame Shift	1.0E-24

2,364	2,367	GK -> EL	GCAA -> AACT	Substitution	Substitution	2.0E-30
2,361	2,362	R -> Q	GT -> AG	Substitution	Substitution	6.3E-36
2,340	2,358	AIDAIRV -> VQKLAAT	CTATCGACGCCATC CGTGT -> TCCAGAAGTTGGCG GCCAC	Substitution	Substitution	4.0E-39
2,336	2,336	R -> S	C -> A	SNP (transversion)	Substitution	2.5E-76
2,333	2,334	N -> V	AA -> GT	Substitution	Substitution	2.5E-76
2,331	2,331	I -> S	T -> G	SNP (transversion)	Substitution	6.3E-80
2,325	2,329	EE -> VR	AAGAG -> TTCGT	Substitution	Substitution	4.0E-71
2,208	2,208		G -> A	SNP (transition)	None	4.0E-191
892	893		GG -> AA	Substitution		6.3E-147
441	442		GG -> AA	Substitution		1.0E-99
312	312		C -> T	SNP (transition)		2.5E-34
K02						
38,175	38,175	V -> A	T -> C	SNP (transition)	Substitution	1.6E-143
37,801	37,801	N -> K	C -> A	SNP (transversion)	Substitution	2.5E-76
29,416	29,416		C -> T	SNP (transition)	None	2.5E-148
18,809	18,809		C -> T	SNP (transition)	None	3.2E-83
12,377	12,377	E -> K	G -> A	SNP (transition)	Substitution	6.3E-130
10,655	10,655	G -> D	G -> A	SNP (transition)	Substitution	4.0E-93
7,965	7,965	D -> N	G -> A	SNP (transition)	Substitution	1.0E-147
7,449	7,449		G -> A	SNP (transition)	None	1.6E-160
5,944	5,944		G -> A	SNP (transition)	None	2.5E-130
892	893		GG -> AA	Substitution		3.2E-144
441	442	V -> A	GG -> AA	Substitution		1.0E-81
K03						
37,801	37,801	N -> K	C -> A	SNP (transversion)	Substitution	1.0E-203
36,318	36,318	V -> A	T -> C	SNP (transition)	Substitution	1.6E-216
36,224	36,224		C -> A	SNP (transversion)	None	6.7E-230
33,192	33,192		G -> A	SNP (transition)	None	3.2E-249
28,242	28,242	H -> Y	C -> T	SNP (transition)	Substitution	6.3E-206
12,429	12,429	T -> I	C -> T	SNP (transition)	Substitution	1.6E-209
10,655	10,655	G -> D	G -> A	SNP (transition)	Substitution	1.0E-224
7,965	7,965	D -> N	G -> A	SNP (transition)	Substitution	1.0E-196

7,449	7,449		G -> A	SNP (transition)	None	4.0E-174
5,944	5,944		G -> A	SNP (transition)	None	1.0E-270
2,208	2,208		G -> A	SNP (transition)	None	1.0E-259
892	893		GG -> AA	Substitution		1.0E-238
K04						
37,801	37,801	N -> K	C -> A	SNP (transversion)	Substitution	7.9E-197
37,729	37,729		G -> A	SNP (transition)	None	4.0E-195
33,192	33,192		G -> A	SNP (transition)	None	1.0E-198
28,242	28,242	H -> Y	C -> T	SNP (transition)	Substitution	1.6E-155
21,116	21,116		A -> G	SNP (transition)		1.6E-143
12,377	12,377	E -> K	G -> A	SNP (transition)	Substitution	4.0E-177
10,598	10,598	R -> H	G -> A	SNP (transition)	Substitution	1.6E-155
5,944	5,944		G -> A	SNP (transition)	None	1.0E-216
892	893		GG -> AA	Substitution		3.2E-207
453	453		T -> A	SNP (transversion)		6.3E-146
K05						
37,881	37,881	V -> A	T -> C	SNP (transition)	Substitution	1.0E-180
37,801	37,801	N -> K	C -> A	SNP (transversion)	Substitution	1.0E-182
24,027	24,027	V -> I	G -> A	SNP (transition)	Substitution	1.0E-126
23,135	23,135		G -> A	SNP (transition)	None	3.2E-116
20,892	20,892	E -> K	G -> A	SNP (transition)	Substitution	4.0E-141
15,809	15,809		G -> A	SNP (transition)	None	1.0E-161
15,340	15,340	A -> T	G -> A	SNP (transition)	Substitution	1.0E-162
14,430	14,430		G -> A	SNP (transition)		4.0E-87
13,082	13,082	A -> T	G -> A	SNP (transition)	Substitution	1.0E-108
11,585	11,585		(G)4 -> (G)3	Deletion (tandem repeat)	Frame Shift	4.5E-109
5,882	5,882	V -> A	C -> T	SNP (transition)	None	4.0E-159

Table S5.4- Common polymorphisms found in the different mutants in relation to wildtype phage.

Phage	Protein	Location	Change	Codon Change	Protein Effect
all mutants	gp40 (tail fiber)	37,801	C -> A	AAC -> AAA	Substitution

KO1, KO2, KO3 and KO4	gp12 (hypothetical protein)	5,944	G -> A	ATG -> ATA	None
KO1, KO2, KO3 and KO4	intergenic region before sigma70 consensus sequence	892	GG -> AA		Substitution
KO1, KO2, KO3	gp17 (DNA ligase)	106,555	G -> A	GGC -> GAC	Substitution
KO1, KO2, KO4	gp14 (primase)	7,965	G -> A	GAT -> AAT	Substitution
KO1, KO2, KO5	gp13 (hypothetical protein)	7,449	G -> A	GAG -> GAA	none
KO1 and KO2	gp19 (DNA polymerase)	13,377	G -> A	GAG -> AAG	Substitution
KO1 and KO3	repeat region	442	GG -> AA		

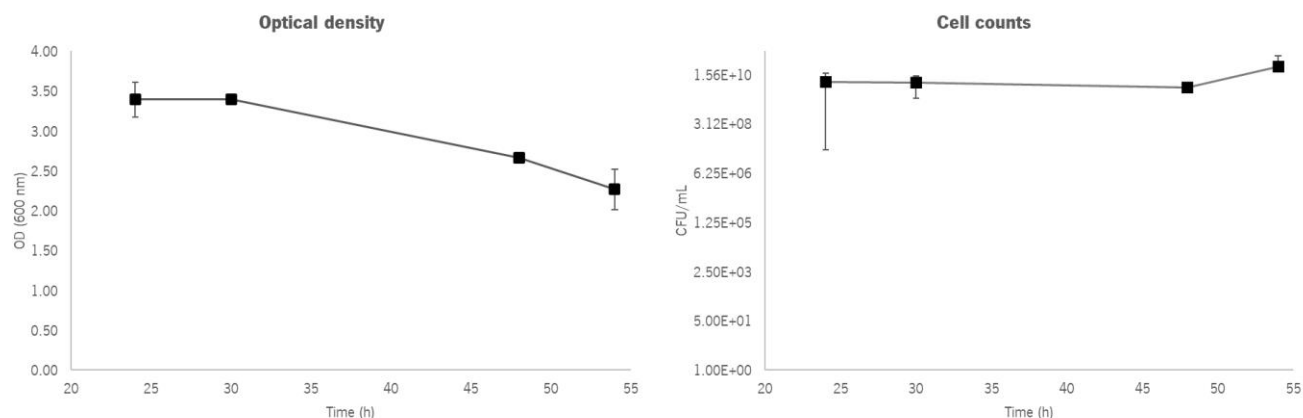


Figure S5.3- Optical density and cell count for *P. aeruginosa* PA01 cultures grown in LB medium at 37°C, with agitation, for 24 to 54h. Two independent assays were performed for each condition with exception of the 48h time point.

Table S5.5- Phage host range of the different phage mutants (KO1, KO2, KO3, KO4, KO5) and wildtype LUZ19 phage in different *P. aeruginosa* clinical isolates. Strains where there is no infection are marked as (-), strains where a inhibition zone is turbid are identified as (T). Strains where a inhibition zone is clear are identified as (C), and strains where it was noticed bacterial resistance (an regrowth of bacteria in the phage-lysed zone) were identified as (RB).

Strain	Phage					
	LUZ19	LUZ19:KO1	LUZ19:KO2	LUZ19:KO3	LUZ19:KO4	LUZ19:KO5
PA01	C	C	C	C	C	C
PA14	-	-	-	-	-	-

1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	T	T	T	T	T	T
6	T	T	T	T	T	T
7	-	-	-	-	-	-
8	T	T	T	T	T	T
9	-	-	-	-	-	-
10	T	T	T	T	T	T
11	RB	RB	RB	RB	RB	RB
12	T	T	T	T	T	T
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	C	-	-	-	-	-
17	C	C	C	C	C	C
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	-	-	-	-	-	-
21	T	-	-	-	-	-
22	-	-	-	-	-	-
23	T	T	T	T	C	C
24	-	-	-	-	-	-
25	-	-	-	-	-	-
26	T	-	-	-	-	-
27	T	-	-	-	-	-
28	T	-	-	-	-	-
29	T	-	-	-	-	-
a22	-	-	-	-	-	-
a62	-	-	-	-	-	-
a63	-	-	-	-	-	-
a64	T	-	-	-	-	-
A65	C	-	-	-	-	-

A66	T	T	T	T	T	T
A67	C	-	-	-	-	-
A69	C	T	T	T	T	T
A70	-	-	-	-	-	-
A71	-	-	-	-	-	-
052e x	T	T	T	T	T	T
064	T	-	-	-	-	-
065	C	C	C	C	C	C
077	-	-	-	-	-	-
078	T	T	T	T	T	T
079	T	-	-	-	-	-
092	C	C	C	C	C	C
144	C	-	-	-	-	-
149	-	-	-	-	-	-
Rmd	C	C	C	C	C	C
Wbpl	C	-	-	-	-	-
Wzy	T	T	T	T	T	T
RmLC	-	-	-	-	-	-
Legend: C-clear; T-turbid; RB-Resistant bacteria; - No halo.						

Table S5.6- EOP of the different phage mutants (K01, K02, K03, K04, K05) and wildtype LUZ19 phage in different *P. aeruginosa* clinical isolates.

Differences in infection between mutants and wildtype are highlighted with colors green for wildtype and red for mutants when the last ones lose the ability to infect compared with LUZ19 phage. Strains in which phages produced isolated plaques are identified as (+), strains where lysis from without occurs are identified as (LW), and strains where there is no infection are marked as (-).

Strain	Phage					
	LUZ19	K01	K02	K03	K04	K05
PA01	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+

8	+	-	-	-	-	-
10	LW	LW	LW	LW	LW	LW
12	+	LW	LW	LW	LW	LW
16	+	-	-	-	-	-
17	+	LW	LW	LW	LW	LW
18	LW	LW	LW	LW	LW	LW
21	+	-	-	-	-	-
23	+	LW	LW	LW	LW	LW
26	LW	-	-	-	-	-
27	+	-	-	-	-	-
28	LW	-	-	-	-	-
29	LW	-	-	-	-	-
A64	LW	-	-	-	-	-
A65	+	-	-	-	-	-
A66	LW	LW	LW	LW	LW	LW
A67	LW	LW	LW	LW	LW	LW
A69	+	LW	LW	LW	LW	LW
052ex	LW	LW	LW	LW	LW	LW
064	LW	-	-	-	-	-
065	+	-	-	-	-	-
078	+	LW	LW	LW	LW	LW
079	LW	-	-	-	-	-
092	+	+	+	+	+	+
144	+	LW	LW	LW	LW	LW
Rmd	+	+	+	+	+	+
Wbpl	+	-	-	-	-	-
Wzy	+	LW	LW	LW	+	+
Legend: LW- Lysis from without; + phage can generate isolated plaques; - No infection.						

Table S5.7- Efficiency of each phage to form plaques on *P. aeruginosa* PA01 lawns incubated at different temperatures.

Temperature (°C)	LUZ19 titer	K02 titer
RT (19-22)	3×10^{10} PFU/mL	6×10^{10} PFU/mL
30	5×10^{10} PFU/mL	5×10^{10} PFU/mL
37	6×10^{10} PFU/mL	3×10^{10} PFU/mL
42	3×10^{10} PFU/mL	4×10^{10} PFU/mL
60	Lawn melted	Lawn melted

CHAPTER VI: FINAL REMARKS AND FUTURE PERSPECTIVES

P. aeruginosa is a dangerous pathogen responsible for life-threatening infections, being very difficult to fight due to its highly resilient nature. Nonetheless, phages have a huge potential as antimicrobial agents against *P. aeruginosa*. A proper understanding of phages' molecular processes is crucial to use these viruses as therapeutic agents. It is particularly important to understand how phages behave in infections-like conditions (e.g., lung environment, *P. aeruginosa* biofilms nature, presence of human tissues/cells, among others), to assure their safety by an in-depth characterization of the role of hypothetical genes encoded in phages' genomes and the mechanisms inherent to the phage life cycle success, and bacterial responses towards phage infection.

Knowledge about phage-host interactions and phage fundamental regulatory processes inspire the advance of several scientific fields, including the development of phage-based antimicrobials, synthetic biology circuits, and biotechnology tools. The application of several advanced techniques (metagenomics, transcriptomics, proteomics, and metabolomics) in phage-host interaction studies is crucial to provide complementary information on general chemical and molecular processes that occur during phage infection.

The work described in this thesis contributed to a better understanding of the interaction between LUZ19-*P. aeruginosa* through transcriptomic and genomic tools. Several specific aspects of this interaction were disclosed such as:

- i) **LUZ19 infection proceeds largely uninterrupted and efficiently, despite the growth limitations imposed by the human cell culture medium and the presence of human lung epithelial cells**, indicating that even under lung infection mimicking-environments the viability of the phage is not affected. This indicates that this phage presents a great potential for application against *P. aeruginosa* associated lung-infections. Nevertheless, studies on phage evolution, pharmacokinetics and pharmacodynamics, and efficacy against chronic/difficult-to-treat *P. aeruginosa* infections should be carefully addressed.
- ii) **The major transcriptional changes occur at the early infection stage**, during the bacterial takeover significant changes are visible in bacterial pathways, related to energy acquisition, transcription, and translation. These specific changes are crucial for phage life cycle progression and seem to be part of a common strategy used by phage, regardless of the growth conditions.
- iii) **Medium-specific responses to phage infection** were also captured, **especially at a regulatory level**. It was hypothesized that these responses arise from phage adaptation of

the bacterial cell to the limitations imposed by human cell culture medium compared to lysogeny broth.

- iv) **The majority of the transcriptomic changes that are common to all growth conditions**, which indicates that phages readily adapt the host machinery towards their needs. In future, full insights on these interactions must be addressed in order to understand how these standard responses are attained by phage, and thus shed light on the function of many ‘viral dark matter’ genes. The disclosure of the role of hypothetical proteins encoded in the phage genome can be a driver of the discovery of genes with novel properties, which can be then applied in several scientific fields.
- v) **Interesting bacterial-specific responses related to human environmental conditions were captured during phage infection**, with particular relevance of an increase in transcription of genes related to virulence and pathogenicity. Yet, the relevance of these changes is to be studied since that many have been reported as a phage strategy to achieve a successful infection. The integration of multiple high-throughput omics is essential to better define if, these are bacterial responses towards phage infection (as mechanisms to thrive and survive phage predation in infection-like conditions) or, if these are unknown mechanisms that phage triggers to guarantee its progeny spread among the bacterial population. As such, the work described in this thesis provides clues towards interesting pathways to study in the future, as well as proves that the study of phage-bacteria interactions can and must be studied under conditions that better simulate lung physiology.

Considering that there is a high demand for functional characterization of phage-bacteria interactions. Transcriptomic studies complemented with genetic engineering approaches can provide an in-depth insight into phage-host interaction. As such, in this work a fast and non-expensive method to generate phages with reduced genomes was employed and demonstrated that:that:

- vi) **The use of selective pressures, such as the increase of temperature and under chelating agents, allows to select phages with smaller genomes which can be used to study their phenotype, associating it with specific changes that occurred at the genomic level.**
- vii) **Mutant phages lacking a substantial portion of the phage genome maintained similar infection parameters and had similar efficiency in killing bacteria (both planktonic and biofilm-associated cells). Nevertheless, their host range was substantially affected as well as the competition at the long term.** This indicates

that probably the mutants are more unstable variants and that have reduced fitness compared to wildtype phages. Perhaps, besides the role of early genes appear to be irrelevant, in long term, these could confer an advantage to phage and probably are particularly important under contexts that are not simulated under laboratory conditions. Therefore, in the future, it must be relevant to assess the phenotype/efficacy of these mutant phages under conditions that, for instance, simulate *P. aeruginosa* infections and compare it with the ancient phage to disclose the relevance in the loss of genomic DNA portions.

To guarantee that the phenotypic observations observed in mutant phages derived from the loss of early genes, these mutants should be obtained without the presence of a mutagenic agent. Thus, this will allow identifying what was the cause of that phenotypic change, the SNPs or deletions of early genes. This information can drive the discovery for instance, of novel anti-bacterial defences that might be codified by the lost early genes, or interesting SNPs that potentially affect phage host range. In the later, the knowledge acquired from SNPs can further provide insights about phage receptors genetic manipulation. This is important, since one of the major trends in phage research biology is to ‘play Lego’ with phage receptors, manipulating them genetically, to extend/target a larger panel of bacterial strains. Not of less importance, should be considered the stability of the mutants since their abundance in phage population can fluctuate over the generations, and thus their evolution must be carefully studied as well as the phenomena through those are naturally generated.

Altogether, the complementation of the information acquired from the in-depth study of phage-bacteria-human cells and synthetic biology will impact phage research, being both important, especially in the phage therapy context to solve MDR associate -infections crisis.