«Study of the lysogenic phages and their potential applications in clinical strains of multi-drug resistant bacteria»

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CERTIIFICAN:

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Y para que así conste, y surta los efectos oportunos, firmamos el presente certificado en A Coruña, 15 de septiembre del 2021.

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CERTIFICATE:

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And for this to be recorded, and take the appropriate effects, we sign this certificate in A Coruña, September 15th, 2021

Directors

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Contents

ABBREVIATIONS

%: percentage

% *w*/*v*: percentage weight/volume

µg: micrograms

µL: microlitres

µm: micrometres

AHL: Acyl-Homoserine Lactone

AMP: AntiMicrobial Peptide

Bci: Bacteriophage Control Infection

BLAST: Basic Local Alignment Search Tool

bp: base pair

BREX: BacteRiophage EXclusion

CA: California

CBD: Cell-wall Binding Domain

CC: Clonal Complex

CD-search: Conserved Domain search

CF: Cystic Fibrosis

CFU: Colony Forming Unit

CHUAC: Complexo Hospitalario Universitario de A Coruña

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

csrA: Carbon Storage Regulator A

CV: Crystal Violet

DISARM: Defence Island System Associated with Restriction-Modification

DMSO: Dimethyl Sulfoxide

DNA: DeoxyriboNucleic Acid

EDTA: EthyleneDiamine Tetraacetic Acid

EOP: Efficiency Of Plating

ESBL: Extended-Spectrum Beta-Lactamase

ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.

GC: Guanine-Cytosine

GEIH: Grupo de Estudio de Infecciones Hospitalarias

GEMARA: Grupo de Estudio de los Mecanismos de Acción y de la Resistencia a los Antimicrobianos

H: hour

HGT: Horizontal Gene Transfer

HSL: HomoSerine Lactone

ICU: Intensive Care Unit

IM: Inner Membrane

INIBIC: INstituto de Investigación BIomédica de A Coruña

LB: Luria-Bertani

LES: Liverpool Epidemic Strain

log: logarithm

LPS: LipoPolySaccharide

M: Molar

MDPI: Multidisciplinary Digital Publishing Institute

MDR: Multi-Drug Resistant

MHB: Mueller-Hinton Broth

MIC: Minimal Inhibitory Concentration

min: minute

mL: millilitre

mM: miliMolar

MMC: MitoMycin C

MOI: Multiplicity of Infection

MRSA: Methicillin-Resistant *Staphylococcus aureus*

NADH: Nicotinamide Adenine Dinucleotide Hydride

NCBI: National Center for Biotechnology Information

NGS: Next Generation Sequencing

nm: nanometre

ºC: degree Celsius (centigrade)

OD: Optical Density

OM: Outer Membrane

OMV: Outer Membrane Vesicle

ORF: Open Reading Frame

PAS: Phage-Antibiotic Synergy

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDR: PanDrug-Resistant

PEG: PolyEthylene Glycol

PFGE: Pulse-Field Gel Electrophoresis

PFU: Plaque-Forming Unit

PG: PeptidoGlycan

PHAST: PHAge Search Tool

PICI: Phage-Inducible Chromosomal Island

PYO: Pyophage

QS: Quorum Sensing

RAST: Rapid Annotations using Subsystem Technology

REIPI: Red Española de Investigación en Patologías Infecciosas

R-M: Restriction-Modification

RNA: RiboNucleic Acid

RND: Resistance-Nodulation-Division

rpm: revolutions per minute

RT-qPCR: Reverse Transcriptase quantitative Polymerase Chain Reaction

SAR: Signal-Arrest-Release

SD: Standard Deviation

SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis

SEIMC: Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica

SERGAS: SERvizo GAlego de Saúde

SMAP-29: Sheep Myeloid 29-amino acid peptide

ST: Strain Type

TA: Toxin-Antitoxin

TEM: Transmission Electron Microscopy

tRNA: transfer RiboNucleic Acid

UDC: Universidade De A Coruña

UK: United Kingdom

UPL: Universal Probe Library

URSS: Union of Soviet Socialist Republics

US: United States

USA: United States of America

WGS: Whole-Genome Sequencing

WHO: World Health Organization

XDR: Extensively Drug-Resistant

ABSTRACT

Bacteria with multiple resistance entail a global threat. In recent years, phage therapy has been widely reconsidered as an alternative to antibiotics. In particular, lytic phages have been shown to have great potential for treating infections with multi-resistant bacteria.

In this thesis, we present the utility and study of lysogenic phages in clinical strains of multi-resistant bacteria. Concerning the potential of lysogenic phages in phage therapy, we have developed the strategy of transforming a lysogenic phage into a lytic one, Ab105-2φΔCI, and characterized its microbial activity. We also have purified and assayed the antimicrobial activity of two endolysins ElyA1 and ElyA2, from two prophages of a clinical strain of *A. baumannii*. Both alternatives have been shown to be effective in combination with antibiotics.

In relation to the study of lysogenic phages in clinical strains, we pointed out the problem of the possible appearance of bacterial resistance against phages and the importance of searching and characterizing these resistance systems by searching *in silico* for phage resistance mechanisms in clinical strains of *A. baumannii*. We also identified 4 complete prophages in clinical strains of *P. aeruginosa*, 2 of them were newly identified: a Siphovirus phage, AUS531phi, and a filamentous Inovirus phage, pf8. Furthermore, we characterized a gene that increases the ability of one of them, the *bci* gene in AUS531phi, to infect the bacteria by the regulation of the Quorum system.

RESUMO

As bacterias con múltiples resistencias supoñen unha ameaza a nivel global. Nos últimos anos, a terapia de fagos reconsiderouse amplamente como unha alternativa aos antibióticos. Especialmente, demostrouse que os fagos líticos teñen un gran potencial para tratar infeccións con bacterias multirresistentes.

Nesta tese presentamos a utilidade e o estudo dos fagos lisoxénicos en cepas clínicas de bacterias con múltiple resistencia aos antibióticos. En relación ao potencial dos fagos lisoxénicos en terapia de fagos, desenvolvemos a estratexia de converter un fago lisoxénico nun lítico, Ab105-2φΔCI, e caracterizar a súa actividade microbiana. Tamén purificamos e caracterizamos a actividade microbiana de dúas endolisinas, ElyA1 e ElyA2 de dous profagos dunha cepa clínica de *A. baumannii*. Ambas alternativas demostraron ser efectivas en combinación con antibióticos.

En relación ao estudo dos fagos lisoxénicos en cepas clínicas, sinalamos a problemática da posible aparición de resistencia contra fagos e a importancia de buscalas e caracterizalas buscando *in silico* mecanismos de resistencia contra fagos en cepas clínicas de *A. baumannii*. Tamén localizamos 4 profagos completos en cepas clínicas de *P. aeruginosa*, 2 deles foron novamente identificados: un fago do tipo Siphovirus, AUS531phi, e outro filamentoso do tipo Inovirus, pf8. Ademais, caracterizamos un xene que incrementa a habilidade dun deles, o xene *bci* en AUS531phi, para infectar a bacteria mediante a regulación do Quorum Sensing.

RESUMEN

Las bacterias con múltiples resistencias suponen una amenaza a nivel global. En los últimos años, la terapia de fagos se ha reconsiderado ampliamente como una alternativa a los antibióticos. Especialmente, se ha demostrado que los fagos líticos poseen un gran potencial para tratar infecciones con bacterias multirresistentes.

En esta tesis presentamos la utilidad y estudio de los fagos lisogénicos en cepas clínicas de bacterias multirresistentes. En relación al potencial de los fagos lisogénicos en terapia de fagos hemos desarrollado la estrategia de convertir un fago lisogénico en uno lítico, Ab105-2φΔCI, y caracterizar su actividad microbiana. También purificamos y caracterizamos la actividad microbiana de dos endolisinas, ElyA1 y ElyA2, de dos profagos de una cepa clínica de *A. baumannii*. Ambas alternativas han demostrado ser efectivas en combinación con antibióticos.

En relación al estudio de fagos lisogénicos en cepas clínicas, hemos señalado la problemática de la posible aparición de resistencia contra fagos y la importancia de buscarlas y caracterizarlas buscando *in silico* mecanismos de resistencia contra fagos en cepas clínicas de *A. baumannii*. También localizamos 4 profagos completos en cepas clínicas de *P. aeruginosa*, 2 de ellos fueron nuevamente identificados: un fago del tipo Siphovirus, AUS531phi, y otro filamentoso del tipo Inovirus, pf8. Además, también caracterizamos un gen que incrementa la habilidad de uno de ellos, el gen *bci* en AUS531phi, para infectar a la bacteria mediante la regulación del Quorum Sensing.

INTRODUCTION

1. ESKAPE pathogens

Nosocomial infections are those infections originated in the hospital environmental by different organisms as bacteria, fungi, virus, parasites and other agents (1).

The nosocomial infections are increasing by the emergence and a growing number of multidrug-resistant bacteria (MDR). Multi-drug resistant, extensively drug-resistant (XDR) and pan-drug resistant (PDR) bacteria are a result of the sum of the acquisition of different gene transfer agents, such as plasmids and bacteriophages containing these genes, or by chromosomic spontaneous mutations (2, 3). Infections caused by MDR, XDR and PDR have been associated with increased length of hospital stay, multiple morbidities, increased cost of hospitalization and high mortality rates (4, 5) The Infection Diseases Society of America have classified a group of nosocomial pathogens as “ESKAPE”, which is an acronym of the species included in the group: *Enterococcus faecium*; *Staphylococcus aureus*; *Klebsiella pneumoniae*; *Acinetobacter baumannii*; *Pseudomonas aeruginosa* and *Enterobacter* species (1). In 2017 the World Health Organization (WHO) published a list of antibiotic-resistant “priority pathogens”, which includes 12 families of bacteria that constitute a threat to human health. This list classifies these pathogens according to the urgency of needs for new antibiotics as critical, high and medium priority. Into the critical wfamily groups are included carbapenem-resistant *Acinetobacter*, *Pseudomonas*, and various *Enterobacteriaceae*, all of them characterized by being a threat in hospitals and nursing houses, and potentially causing severe and often deadly infections.

1. a. *Acinetobacter baumannii*

Acinetobacter baumannii belongs to the genus *Acinetobacter*, which comprises a heterogeneous group of immobile, large bacillus or coccobacillus, strictly aerobic, non-fermentative glucose, catalase-positive, oxidase-positive and has a guanine and cytosine content between 39% and 47% (6). Species within this genus can be found in a wide variety of environments such as water, air and human skin (7). The most clinically relevant species within this genus are *A.*

baumannii, *Acinetobacter pittii* sp nov. and *Acinetobacter nosocomialis* sp. nov. Together with *Acinetobacter calcoaceticus* form a common group called *A. calcoaceticus-A. baumannii* complex (8, 9).

On the contrary to other species of the genus *Acinetobacter*, that are frequently isolated from soil, water and animals, *A. baumannii* is almost exclusively isolated from hospital environments, particularly in Intensive Care Units (ICUs) (10). In the last 30 years, *A. baumannii* has reached a wide distribution among the developed countries in the hospital environment and is considered one of the most important MDR bacteria causing nosocomial infections (6).

The success of *A. baumannii* in a hospital environment is due to various causes such as persistence for long periods (resistance to desiccation), resistance to antimicrobials and disinfectants or biocides and virulence factors (such as biofilm formation, adhesion mechanisms and iron acquisition). The resistance and virulence genes are mainly acquired via mobile elements as plasmids, bacteriophages and transposons (11-14). All these causes contribute to the nosocomial outbreaks caused by *A. baumannii* being very difficult to control (15, 16). As a result of the II Spanish Multicentric Study (GEIH-REIPI *Acinetobacter baumannii* 2000-2010), 654 clinical isolates were obtained from 42 participant hospitals and it was established that the resistance to carbapenems (82-86% vs 43-48%), ceftazidime (99% vs 83%), sulbactam (65% vs 53%) and colistin (3% vs 0%) has grown significantly from 2000 to 2010 (17). *A. baumannii* causes serious infections such as bacteremia, meningitis and pneumonia in immunocompromised patients in ICU units (18).

1. b. *Pseudomonas aeruginosa*

Members of the genus *Pseudomonas* are rod-shaped bacillus, gram-negative, with one or more flagellums that provide motility, generally aerobic, non-spore-forming, catalase-positive and oxidase-positive (19). Its genome has a size between 5-7 Mbp. *P. aeruginosa* is present in diverse environments and is capable of tolerating a great variety of physical conditions. In hospital environments, *P. aeruginosa*, can be isolated from a variety of sources like respiratory therapy equipment, antiseptics, soap, sinks, etc. and in community

populations, it can be found in swimming pools, whirlpools, contact lens solutions, etc. (20).

Horizontal gene acquisition confers opportunistic pathogen *P. aeruginosa* strains the ability to acquire foreign genetic material, such as plasmids. For example, this process grants the bacteria with antibiotic-resistant genes encoding β-lactamases (bla), such as extended-spectrum β-lactamases (ESBL) (21). Furthermore, the highest ubiquity, prevalence and persistence of *P. aeruginosa* in clinical environments are due to its exceptional capability of survival and adaptation by obtaining a wide number of regulatory and controlling factors (22) as quorum sensing (QS), a communication system that allows bacterial populations to control widespread processes crucial for bacterial adaptation and survival, such as the regulation and control of cellular secretion systems (23, 24). QS system controls the pathogenesis of many organisms through regulating gene expression of several virulence factors, including motility, biofilm formation or pyocyanin secretion in *P. aeruginosa* (25). The knowledge that these virulence factors are under the control of the QS system leads researchers to search for anti-QS compounds in biofilm-associated chronic infections (26).

P. aeruginosa is the second leading cause of nosocomial pneumonia (and the leading cause of pneumonia among pediatric patients in the ICUs), the third most common cause of urinary tract infections, the fourth-most frequently isolated pathogen in surgical site infections and the seventh leading contributor to bloodstream infections (27-29). It is also the main cause of morbidity and mortality in patients suffering from cystic fibrosis (30-32). Infections caused by *P. aeruginosa* can be classified as: 1) acute superficial infections in immunocompetent patients, 2) acute invasive infections in patients with significant comorbidities or immunodepression and 3) chronic infections (33).

Cystic fibrosis (CF) is a life-threatening, autosomal recessive disease that causes persistent lung infections and increasingly limits the ability to breathe Patients with CF experience multiple bacterial infections during their lives. There is a strong antibody response in the lungs, although for the immune system remains difficult to eliminate bacterial infections (34). A patient with CF

will have up to 10 times more inflammation when a pathogen enters the lung, especially in neutrophil recruitment (35). Infections caused by *P. aeruginosa* are the most prominent in CF adult patients due to the multiple virulence factors that enable evasion of the host response, for example, pyocyanin production, biofilm production or flagellum, all of them regulated by QS system (36).

2. Bacteriophages and Phage Therapy

2. a. Phage therapy history

Bacteriophages (phages), viruses that infect bacteria, are the most ubiquitous organisms on Earth, playing the main role in preserving the microbial equilibrium on this planet (37). Phages have an obligate requirement for a host and have diverse life cycles. Felix d'Hérelle in 1917 was a pioneer using the bacterial killing ability of phages as a therapeutic strategy against bacterial infection (38). As D'Hérelle is considered the discoverer of phage therapy, Frederick Twort described the bacteriophages for the first time in 1915 in a bacterial culture (39).

The application of phages has been proposed as a therapy to treat acute and chronic infections because of the specificity of bacterial target hosts. The interest in phage therapy as a treatment for bacterial diseases in the pre-antibiotic era was enormously high. In fact, the only therapy available in the 1920s and almost in the 1930s for some bacteria such as pneumococci and diphtheria was the use of antibodies through serum therapy and all-new alternatives were well appreciated (40, 41). However, the therapeutic use of phages to treat bacterial infection was highly controversial from the very beginning and not generally accepted by the public or the healthcare community. Thus, The Council on Pharmacy and Chemistry of the American Medical Association, concluded in 1934 that due to the lack of appropriate controls, inconsistent results and the deficiency of reproducibility by the early studies the evidence for the therapeutic value of the phages was unconvincing (42). The emergence of antibiotic chemotherapy in the 1930s and later penicillin in the 1940s decreased the enthusiasm for phage research and it was demoted to eastern countries, where research and development remained active (40). The increased availability of antimicrobial drugs after the Second World War

further contributed to the low appreciation of the bacteriophages and the establishment of antibiotics as the regular treatment for bacterial infections.

The majority of the knowledge about phage therapy comes from eastern Europe. The Eastern Bloc (Poland, the URSS and Georgia) were the historical continuators of phage therapy during the XX century. The Eliava Institute of Bacteriophages (Tbilisi, Georgia), which was the key reference in the history of phage therapy, is one of the major sources of information about bacteriophages with the Hirszfeld Institute (Wroclaw, Poland) (43, 44). Phage therapy persists in the whole URSS because of their early scientific interest in the 1920s, the treatment of dysentery, wound infections and cholera in World War II and the synergy between the establishment of the phage therapy during the Cold War and the basic research in diverse countries of URSS like Georgia (43, 45).

Actually, due to the emergence of the MDR microorganisms, the search for new antimicrobial agents has developed a renewed interest in phage therapy, which includes the use of phages alone or in combination as phage cocktails, or the use of products derived from phages as are the endolysins, enzymes that targets and degrades the bacterial peptidoglycan cell wall layer.

2. b. Bacteriophage life cycle

The infection by bacteriophages starts with the attachment to a specific receptor in the bacterial surface and the sequent injection of the genetic material into the cell. The lytic cycle follows a process with 5 steps (Figure 1):

1. Attachment: After recognizing, tail proteins bind to a specific receptor, through electrostatic interactions, on the bacteria surface. Bacteriophages target a range of host cell wall molecules or protruding structures, such as membrane proteins or pili.
2. Penetration: The phage injects its genetic material through the cell membrane into the host cell. This process is coordinated with the attachment by the baseplate of the phage (46).
3. Phage replication: Once inside the cell, when a lytic cycle occurs, phage produces endonucleases and exonucleases necessary to degrade the host genome. The host cell provides the necessary machinery to

replicate the phage genetic material and produce the phage proteins (47).

4. Assembly: The phage proteins are organized to form the phage structure and to pack the genetic content of the progeny.
5. Lysis: Once the progeny phages are assembled, the endolysins and holins produced by the phage, lyse the host cell peptidoglycan releasing the phage progeny and killing the host cell. Afterwards, the phage can infect and reproduce the same life cycle in neighbour bacteria. This huge progeny production is an advantage for phage therapy, as the number of phages is increased in the infection site (48).

Phages that can undergo a lysogenic cycle (**Figure 1**) are known as temperate or lysogenic phages. In the lysogenic cycle, temperate phages insert their DNA into the host chromosome at specific sites with the help of phage-encoded integrases (49). Once integrated into the host genome, the bacteriophage, known as prophage in this state, can remain integrated for generations, but they can be switched at any time to the lytic cycle by a process known as induction (50). The lysogenic cycle can last indefinitely until the bacteria are exposed to unfavourable stress conditions, such as antibiotic treatment, oxidative stress or DNA damage (51), or it can occur spontaneously at a low frequency (52). Furthermore, prophages can influence the induction of other phages (53, 54). After the induction, prophages can replicate episomally or by transposition. Later, the virion particles are assembled and packaged with the phage DNA, beginning the lytic cycle by breaking the cell wall and being released to the environment.

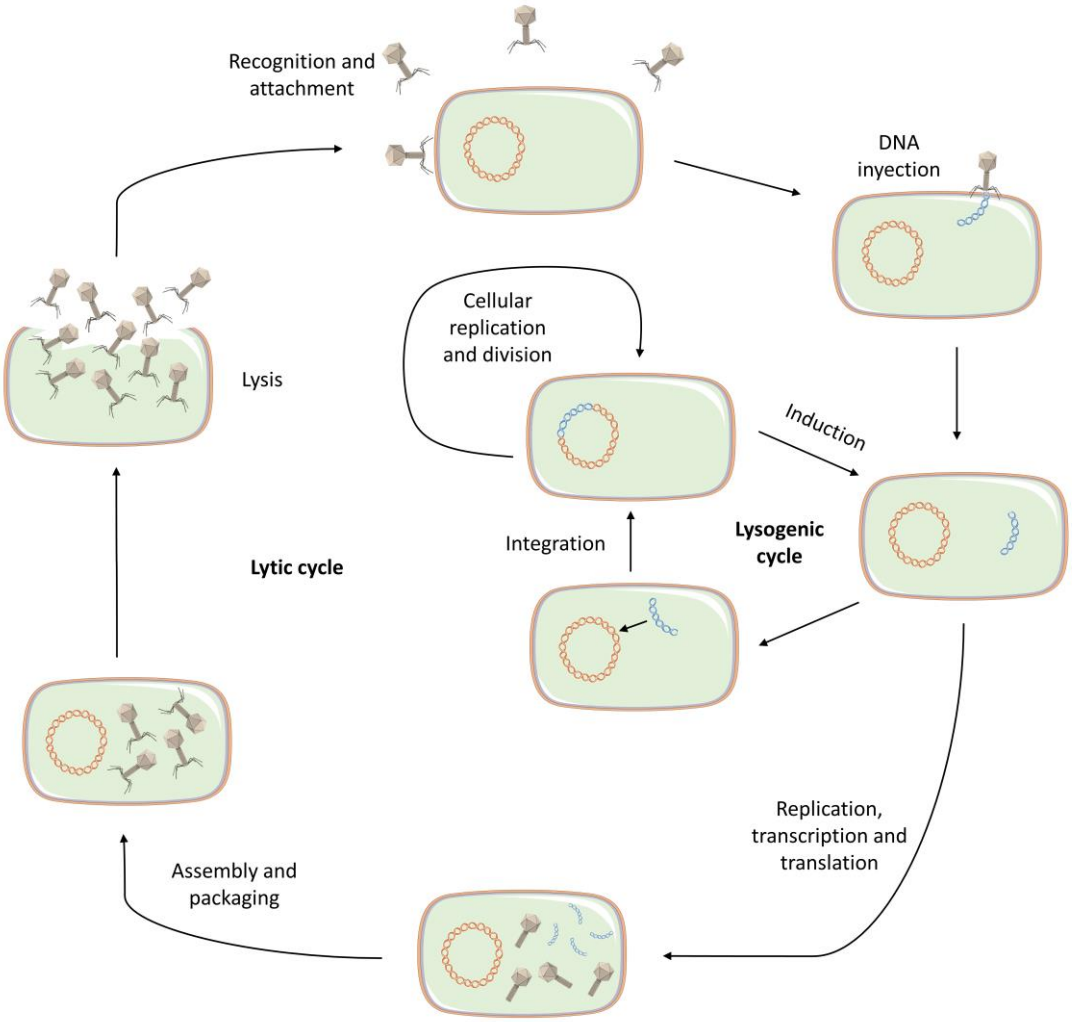

Figure 1. Bacteriophage life lytic and lysogenic cycles.

One model of the lysogenic cycle is the life cycle of the λ phage (**Figure 2**) (55). It was the best model to understand the gene regulatory mechanisms that give place to the transition from lysogeny to lytic development (56). The Cro-CI system, which regulates this transition, is known since the 1960s in λ phage. The feedback between *cI* and *cro* genes switches the lytic-lysogenic life cycle of the bacteriophage. When a bacterial host suffers any damage provoked by a stress situation, Cro protein accumulates and activates promoters for the DNA phage replication and consequent bacterial cell lysis. In a normal "healthy" environment, Cro synthesis is low, so CI protein accumulates and activates promoters for phage integration into the host chromosome, starting a lysogenic cycle (57-60).

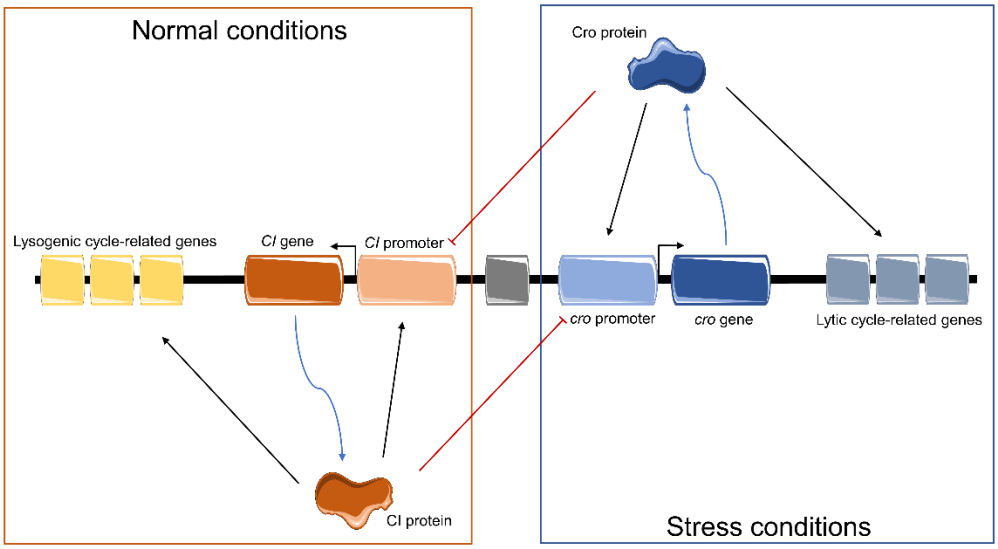

Figure 2. Mechanism of control of the lysis-lysogenic cycle in the λ phage model.

Nevertheless, the mechanism of induction is not perfect and prophages can sometimes leave behind parts of their DNA or take portions of host DNA with them when they re-circularize, transporting bacterial genes (such as antibiotic resistance genes, toxin/antitoxin and other virulence-related genes) from one strain to another by transduction (61). Recently, a study in phages that infect *Bacillus* species has demonstrated that the transition between lytic and lysogenic infection is dependent on small peptides produced by phage in a similar process to quorum sensing (62).

An example of lysogenic phages is filamentous bacteriophages. Recent studies have been conducted on the presence of lysogenic filamentous phage from the *Inoviridae* family in many bacterial species. This type of phages undergoes a non-lytic lifecycle in which they episomally replicate producing large numbers of progeny phage, not killing bacteria, but keep releasing viral particles. Many have been revealed to affect the host's behaviour, resulting in favourable consequences on the host and, consequently, on the phage (63). Some of these host-phage relationships are the increase in biofilm formation, cell motility, the expression of toxins or even conferring advantages to the bacteria in non-favourable conditions (64-66). In *P. aeruginosa*, they are recognised as Pf phages and are distributed among clinical strains, especially in infected

cystic fibrosis patients, conferring these strains evolutionary advantages such as biofilm formation, antibiotic resistance and motility (67).

3. Phage therapy

Theoretically, there are no bacteria that cannot be lysed by at least one bacteriophage (68). The main advantage that antibiotics have against bacteriophages is their large spectrum of activity. But bacteriophages have several advantages over antibiotics, as they have a narrow spectrum of activity, protecting the normal microbiota, they can multiply in the infection site, are abundant in nature and have a low-cost production (69). Bacteriophages are significantly safer, well-tolerated and their administration is easier because and they can remain in the human body for relatively prolonged periods (68). Furthermore, bacteriophages can be engineered to overcome some limitations of the antibiotic treatment, taking advantage of the new cost-effective and large-scale DNA sequencing and DNA synthesis technologies (70).

It has been developed several techniques to detect and characterize phages with therapeutic potential. Methods like spot testing, plaque testing, culture lysis and routine test dilution are used to determine the different properties of the bacteriophage infection for developing the desired phage therapy (71):

- Phage virulence: the ability to completely lyse a bacterial culture, taking into account the productivity of the phages to kill bacteria faster than their growth and the appearance of bacterial-resistance mutants.
- An obligated lytic growth or the search for genes adequate for therapies like endolysins.
- Host range: number of target pathogen species that a single bacteriophage has.
- Other properties not intrinsically essential for phage therapy but necessary for phage characterization:
    - The efficiency of plating (EOP). A titer of the phage on a given bacterial cell line compared to the maximum titer observed.
    - Morphology of the phage determined by electron microscopy.

- Whole-genome sequence (WGS) provides a wide range of information: lack or presence of toxin genes, the ability to form a lysogen, phylogenetic characterization, etc.
- Adsorption curve, for measuring the adsorption rate at which each virion in the environment is attached to the bacteria.
- One-step growth curve, for calculating the burst size, which is the number of virions released per bacterium.
- Pulse-field gel electrophoresis (PFGE) to measure the size of a phage genome.

Phage therapy could be performed as a single phage application or with a phage cocktail formed by a mixture of phages (72). Monophage therapy is usually proved for implementing a general phage therapy with other antimicrobial agents, but in some circumstances, they have a sufficiently wide host range to being applied alone. The main advantages of this type of therapy are the simplification of the treatment due to the isolation and purification of a single phage, and the reduced possibility of subject phage immunological response. Monophage therapy has been used in pneumonia infections by *A. baumannii* carbapenem-resistant and in mice models to treat imipenem-resistant *P. aeruginosa* bacteriemia (73-75). However, the use of monophage therapy in clinical practice may be disturbed due to the quick appearance of phage-resistant bacterial. Evolution shows that phage is able to overcome bacterial resistance, although this may not be effective enough to apply monophage therapy in clinical usage. An additional disadvantage of using a single phage is the accurate match that should have the pathogen and the phage (76).

To sort out the monophage therapy disadvantages, especially to avoid the appearance of phage-resistant bacteria, “polyphage” therapy or cocktail phage therapy has been developed and more used in clinical trials rather than the single phage treatment (77). The design of phage cocktails required more complex preparation and purification processes, resulting in a higher cost of time and money and unpredictability of pharmacokinetic and pharmacodynamic properties (72). Despite this outcome, this design could be focused on targeting a single bacterial strain, multiple strains or even multiple species. Furthermore,

to limit the previously commented outcomes, individual phages of a cocktail can be tested to check their activity (78).

Phage cocktails have been used in western countries as commercial preparations. For example, Pyophage (PYO) is a commercially available phage preparation by related to Eliava Institute company Eliava Biopreparations (http://phage.ge/products/pyo-bacteriophage/). It is composed of a cocktail of phages that target *S. aureus*, *Streptococcus spp.*, *Escherichia coli*, *P. aeruginosa* and *Proteus* species.

In 2019, a 15-year-old patient with CF, comorbidities and failure for lung transplant received for first-time treatment with genetically engineered phages, against a *Mycobacterium abscessus* infection (79).

But as phages have many advantages, they also have a disadvantage similar to antibiotics, as the emergence of phage resistant bacteria, which can be solved through different ways as with the use of phage cocktails because of the recognition of different bacterial receptors for the attachment, the use of enzymes derived from phages as endolysins and the use of a combination of phages and antibiotics with a synergic effect (80, 81). One of the disadvantages of phage therapy is the bacterial development of phage resistant mechanisms (68). Bacteria can turn resistant to phages through chromosomic mutations and specific resistant mechanisms (82). In recent years, many phage resistance mechanisms have been discovered and characterized to help the bacteria to survive phage infection. The emergence of phage-resistant bacteria is associated with spontaneous mutations and adaptation, especially modifications in the receptors which phages use to adhere to the bacterial membrane (82). The principal mechanisms are related to different phases of the infection, for example, the blockage of the DNA injection through outer membrane vesicles or the action of superinfection exclusion systems promoted by the genes encoded in the prophages presents in the bacterial genome (83,84).

3. a. Endolysins

Phage endolysins are cell wall hydrolytic enzymes used to break the peptidoglycan of phage-infected bacteria and release the phage progeny assembled in the cytoplasm (85). In a first approach, endolysins are more

effective against gram-positive bacteria than in gram-negative bacteria, where the cell wall is surrounded by the outer membrane, which acts as a barrier to the endolysins preventing its access to the peptidoglycan layer (86), and for this reason, endolysin research has mostly focused on gram-positive bacteria. There have been reported several endolysins that kill gram-positive bacteria, mainly on *Streptococcus* sp., *S. aureus* and *Bacillus anthracis* (87-90).

However, endolysins have been turned into a potential treatment for gram-negative bacteria under certain conditions (91). In the gram-negative phages, endolysins are part of a host lysis process of three steps, corresponding to the destabilization of each of three layers of the cell envelope: inner membrane (IM), peptidoglycan (PG) and outer membrane (OM) (92) (**Figure 3, A**). The model is described using the phage lambda system (93, 94).

The phage exit from the cell implies the activity of three proteins, holins, spanins and endolysins. In the first step of the cell lysis, holins accumulate in the host cell cytoplasmic membrane forming oligomeric states. At a specific time, the lysis process starts and the holin forms non-specific channels resulting in membrane depolarization and allowing the endolysin to break the peptidoglycan (**Figure 3, B**) (95-98). Finally, the spanins fuse removes the OM by fusing it with the IM (**Figure 3, D**) (99).

Endolysins come into play in the third step, reaching the peptidoglycan and degrading it through muralytic activity (**Figure 3, C**). There are two functional types of endolysins depending on the absence or presence of a signal sequence: canonical endolysins and signal-anchor-release endolysins (SAR).

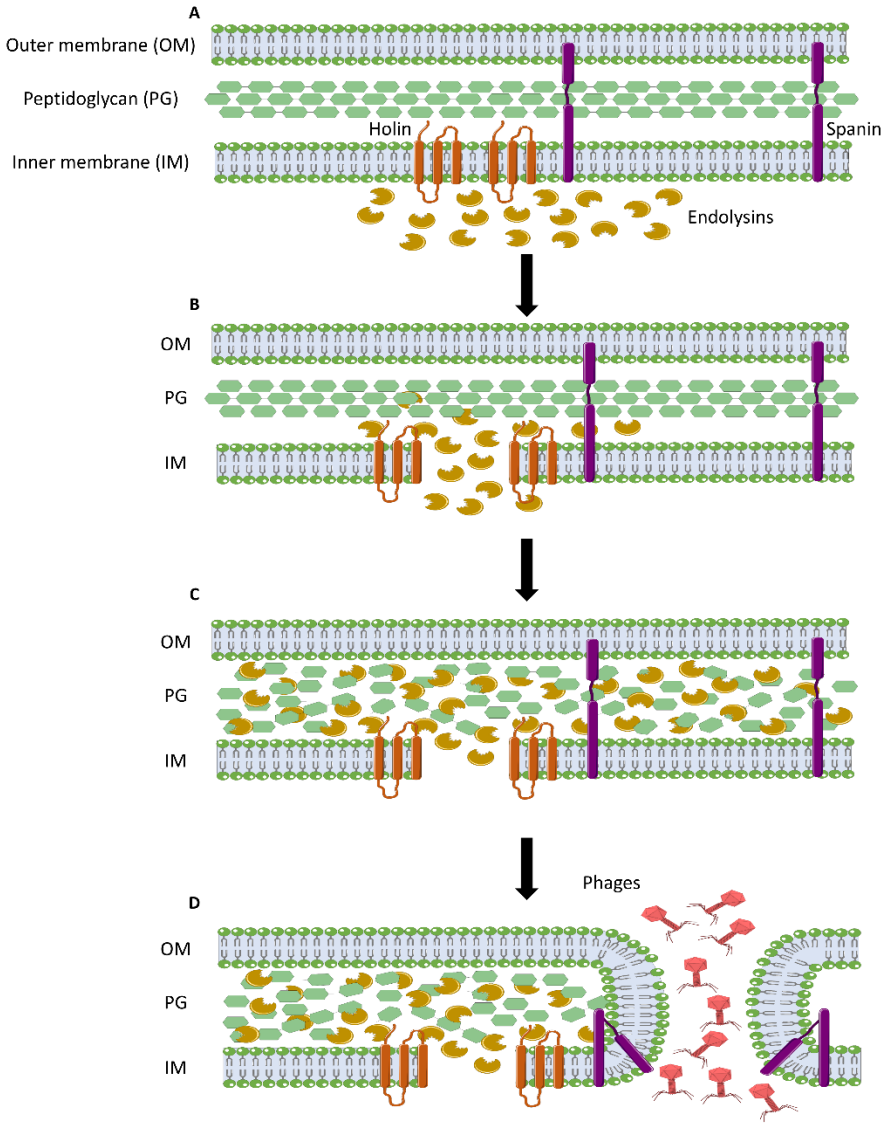

Figure 3. Phage lysis and exit process

The first endolysins were described in the literature in the 1960s-70s in T-phages infecting *E. coli* (100, 101). They were named 'lysozymes' due to their similarity catalytic specificity to the human lysozyme, although today this name is reserved for its originally described activity (102, 103). Because of the variation in cell wall composition between gram-negative and gram-positive, endolysins against each group of bacteria show differences in their architecture (86, 104, 105). Despite having a conserved biological function, endolysins are very diverse in structure: they can have a globular (with a single enzymatically active domain) or a modular structure (at least two modules, most of them with a cell wall binding domain) (106). There are different catalytic specificities found

in endolysins apart from lysozyme, such as transglycosylase, glucosaminidase, amidase or endopeptidase activity (107).

Regarding safety and future approaches of endolysins, their specificity is considered beneficial as it does not harm the normal microflora of the host in comparison with antibiotics. A 2018 preclinical study did not show changes in microarray and gene profile of human cell lines exposed to endolysins Cpl-1 and Pal-1 for six hours (108). One of the most profitable advantages of endolysins is that bacterial endolysin resistance development is very low or inexistent. This should be because of the historical coevolution between bacteria and phage: the development of endolysin resistance must be linked to the modification of the cell wall highly conserved structures and their modification is assumed to be detrimental to the host organism (105, 109). Moreover, acting on the cell wall without entering the bacterial cell, endolysins avoid the typical resistance mechanisms to antibiotics (e.g., decreased permeability, efflux pumps) (110). In addition, the capacity to hydrolyse different bonds in the peptidoglycan layer of some endolysins, is due to the presence of two catalytic domains, thus reducing the possibility of the bacteria developing endolysin resistance (111, 112).

Until present days, a wide variety of assays have shown the therapeutic ability of endolysins in the battle against infections of multidrug-resistance microorganisms. Nevertheless, certain endolysins present some drawbacks, such as limited *in vivo* half-life, inflammatory cytokine development and antibodies neutralization (113, 114). Most of the efforts have been focused on the combined use of the endolysins with permeabilizing substances to pass the outer membrane in gram negatives, although some endolysins have the intrinsic capacity to pass this membrane (115). Data from clinical trials on potential endolysins can also provide a different opportunity for professionals to combat multidrug-resistant bacteria, which are often difficult for conventional antibiotics to eliminate (116). Although a narrow number of endolysins have entered into clinical trials, there are some of them commercially available (117). Moreover, it has been proved that genetic engineered endolysins display similar lytic effects to their native counterparts when they are applied exogenously to susceptible bacteria (104, 118).

The vast majority of the studies about endolysins have been developed in gram-positive bacteria such as *Streptococcus pneumoniae* and *S. aureus*, most of them with *in vitro* biofilm models and *in vivo* sepsis models (119-126). In *A. baumannii* there have been discovered several endolysins. The first discovered was LysAB2 endolysin, which has a broad activity range against gram-positive and gram-negative bacteria (127). LysSS is a recombinant endolysin that shows activity mainly against *A. baumannii* and *P. aeruginosa*, but also against *E. coli*, *K. pneumoniae*, *Salmonella* and *S. aureus* (128). PlyF307 is the first gram-negative endolysin to show *in vivo* effectivity in a murine bacteremia model (129). PlyAB1 and Ply6A3 endolysin have displayed a highly specific lytic effect against clinical multidrug-resistant *A. baumannii* isolates (130, 131). Whole-genome sequencing is a key tool for the discovery of potential endolysins, such as LysAB3 and LysAB4 (132, 133). LysAB3 endolysin has been tested to degrade *A. baumannii* biofilms *in vitro* (134). Combination with antibiotics like colistin has resulted in a successful synergistic effect of the LysABP-01 endolysin (135). In 2021, two novel endolysins from *A. baumannii* has been discovered: LysAB54 which also shows bactericidal effect against *P. aeruginosa*, *K. pneumoniae* and *E. coli*, even though in absence of outer membrane permeabilizers (136); and Abtn-4, effective against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *Enterococcus* and *Salmonella*. In *P. aeruginosa*, the application of endolysins such as EL188, KZ144 or OBPgp279 has been reported (106, 137, 138). LysSAP26 is a recent genetically engineered endolysin with a wide broad spectrum that has been shown effective against carbapenem-resistant *A. baumannii*, *E. coli, K. pneumoniae* and *P. aeruginosa*, as well as to oxacillin-resistant *S. aureus* and vancomycin-resistant *E. faecium*, with promising results targeting both gram-negative and gram-positive bacteria (139).

3. b. Phage therapy in combination with antibiotic therapy

To avoid the limitations of phage therapy, the combination of phages with antibiotics shows a promising synergistic effect to act more effectively (140-142). Several studies have proved the beneficial results of the combination of phages and antibiotics, called phage-antibiotic synergy or PAS. Also, a combined treatment can lead to the restoration of antibiotic sensitivity, for

example when the bacteriophage receptor is in the bacterial drug efflux systems (143). Tail proteins of phages recognise, in a specific way, bacterial receptors, which usually are membrane proteins or LPS that at the same time are the target for several antibiotics (144). For example, bacteriophage OMKO1 is able to recognise the outer membrane porin M (OprM) and increase the bacterial sensitivity to several antibiotics due to the changes in the efflux pump mechanism of the OprM in *P. aeruginosa*. It was also reported the reduction in the formation of bacterial biofilms by the synergistic effect of these combinations (145, 146). Due to the wide diversity of phages, there are a great variety of combinations. For example, an outstanding alternative to personalize therapies for individuals is to combine a phage cocktail therapy with different antibiotics (147).

The combination of phages and antibiotics has the advantage of exerting two different selective pressures that are likely to be more effective than only one alone (80). Phage-antibiotic combinations tend to be especially relevant where there are limited antibiotic alternatives due to multidrug resistance or whether there is restricted dissemination of antibiotics in the infected area of interest. An advantage of this combination is the selective pressure that phages enforce on certain strains of bacteria. It has been proved that this evolutionary selection may cause a cost in the fitness of bacteria, retrieving them a sensitivity to antibiotics (143). One example of the fitness trade-off for phage resistance is the spontaneous mutations in Epa exopolysaccharide biosynthesis genes in *E. faecalis* give resistance to lytic phage infection but increases sensitivity to cell membrane-targeting antibiotics (148, 149). Another example is the development of sensitivity to polymyxin B in addition to a reduction in virulence in *Y. pestis* in phage-resistant bacterial strains due to the truncation of the LPS lengths (150). Furthermore, phages use multidrug efflux pumps as receptors. It has been proved that mutations in these molecules in *P. aeruginosa* produced by phage selective pressure alter the pump mechanism, leading to re-sensitization of antibiotics in which resistance depends on these pumps (143). In *A. baumannii*, the emergence of phage resistance mechanisms has been proved to be related to the increment of colistin sensitivity (151).

Phage-antibiotic combination is also effective against biofilms: phage produces enzymes, such as depolymerases and endolysins, which can break the extracellular matrices of biofilms thus facilitating the antibiotic action (152). For example, Chaundry et al. demonstrate that PAS activity was noted after a biofilm growth when a combination of phages and antibiotics was present in *P. aeruginosa*, unlike either agent was used alone (146).

Endolysins hold promise as individually applied antibacterials, but they also have been revealed to act synergistically in combination with other antimicrobial agents. The effectiveness of antibiotics can be restored by using a combination of endolysins and antibiotics. For example, Daniel et al., demonstrated in an *S. aureus* MRSA-infected mice model, a duplication of the survivor number when the endolysin ClyS was combined with oxacillin, even reducing the ClyS endolysin dose (153).

There are many studies *in vitro*, *in vivo* and case studies with phages and antibiotics whose synergy was enough to demonstrate an effect in the bacterial infection (**Table 1**). A large number of the synergistic combination of phage with antibiotics were focused on *P. aeruginosa* because of its clinical impact involved in cystic fibrosis, hospital-acquired pneumonia or urinary tract infections (154).

Pathogen	Model	Phage+antibiotic	Reference
Acinetobacter baumannii	*in vitro*	LysABP-01*+colistin	Jansen et al. 2018 (135)
	Case study	φIV cocktail + minocycline	Thummeepak et al. 2016 (155)
	in vitro	vB_AbaM-KARL-1+meropenem/ciprofloxacin/colistin	Schooley et al. 2017 (156)
	Case study	cocktail (ϕAbKT21phi3, ϕKpKT21phi1) + colistin/meropenem	Nir-Paz et al. 2019 (157)
	in vitro	Aba-1, Aba-2, Aba-3, Aba-4, Aba-6) + ciprofloxacin, levofloxacin, trimethoprim/sulfamethoxazole, gentamicin, netilmicin	Grygorcewicz et al. 2020 (158)
Pseudomonas aeruginosa	*in vitro* and *in vivo* (mice)	Pf1, Pf3 + carbenicillin/gentamicin/tetracyclin-chloramphenicol	Chan et al. 2018 (159)
	Case study	Cocktail of 6 pyophages + meropenem/colistin	Chaudhry et al. 2017 (160)
	in vitro	σ-1 + ceftriaxone	Lin et al. 2018 (161)
	in vitro and *in vivo* (rats)	vB_PsaP PAT14 + Imipenem-cilastatin/amikacin	Uchiyama et al. 2018 (162)
	in vitro	LUZ7 + streptomycin	Hagens et al. 2006 (163)

	in vitro	NP1, NP3 + ceftazidime/ciprofloxacin/colistin/gentamicin-tobramycin	Knezevic et al. 2013 (146)
	in vitro	OMKO1 + Ceftazidime/ciprofloxacin/tetracycline-erythromycin	Torres-Barceló et al. 2014 (143)
	in vitro and *in vivo* (rats)	PP1131 + ciprofloxacin	Chan et al. 2016 (164)
	Case study	OMKO1 + Ceftazidime	Yilmaz et al. 2013 (165)
	in vitro	PEV20 + ciprofloxacin/amikacin/aztreonam/colistin-tobramycin	Oechslin et al. 2017 (166)
	in vitro	KPP21 + cefoperazone, chloramphenicol, fosfomycin, moxalactam, meropenem. KP22 + amikacin, aztreonam, cefepime, cefoperazone, cefoperazone/sulbactam, cefozopran, ceftazidime, cefotaxime, gentamicin, meropenem, moxalactam, piperacillin, tobramycin. KP23 + cefoperazone/sulbactam, fosfomycin, tobramycin	Torres-Barceló et al. 2018 (167)
	in vitro	LKD16, LUZ7, 14/1, EL + Ceftazidime/ciprofloxacin	Akturk et al. 2019 (168)
	in vitro	EPA1 + gentamycin/ciprofloxacin	Khawaldeh et al. 2011 (169)
	in vitro	vB_PaeP_4024 (ϕ24), vB_PaeP_4054 (ϕ54) + ciprofloxacin	Luscher et al. 2020 (170)
	in vitro and *in vivo* (mouse)	PAM2H cocktail + ceftazidime, ciprofloxacin, gentamicin, meropenem	Engeman et al. 2021 (171)
	in vitro	vB_PaeAM.P2 (AMP2) + ciprofloxacin	Menon et al. 2021 (172)
	in vitro	E79, phiKZ + AzLys (antibiotic aztreonam lysine)	Davis et al. 2021 (173)
Klebsiella pneumoniae	*in vitro*	B5055 phages + amoxicilin	Bedi et al. (2009) (174)
	in vitro	KPO1K2+ciprofloxacin	Verma et al. 2010 (175)
	Case study	ϕAbKT21phi3/ϕKpKT21phi1 + meropenem/colistin	Nir-Paz et al. 2019 (157)
Escherichia coli	*in vivo* (chicks)	SPR02/DAF6 + enrofloxacin	Comeau et al. 2007 (176)
	in vitro	ΦMFP+cefotaxime-aztreonam-cefixime-ceftriaxone-ceftazidime/gentamicin/tetracyclin; RB32-33, T3, T7 + cefotaxime; T4 + cefotaxime-piperacillin-ampicillin-ticarcillin/nalidixic acid/mitomycin	Ryan et al. 2012 (177)
	in vitro	T4+cefotaxime	Coulter et al. 2014 (178)
	in vitro	T4+tobramycin	Valério et al. 2017 (179)
	in vitro	ECA2+ciprofloxacin	Huff et al. 2004 (180)
	in vitro	gT0E.co-MGY2 + ampicillin	Moradpour et al. 2020 (181)
	in vitro and *in vivo* (Zebrafish and mice)	ΦEcSw + kanamycin, chloramphenicol, ampicillin	Easwaran et al. 2020 (182)
	in vitro	φSZUT, φSZIP1, φSZIP2 + amoxicillin, ampicillin, cefadroxil, ciprofloxacin,	Iqbal et al. 2020 (183)

		chloramphenicol, cefixime, tetracycline	
	in vitro	ΦHP3 + trimethoprim, ciprofloxacin, colistin, ceftazidime, kanamycin, chloramphenicol	Liu et al. 2020 (184)
	in vitro	HK97 + ciprofloxacin	Al-Anany et al. 2021 (185)
Enterococcus faecalis	*in vitro*	EFDG1, EFLK1 +ampicillin	Khalifa et al. 2018 (186)
Enterococcus faecium	*in vitro*	113 (ATCC 19950-B1) + daptomycina, ampicilina, ertapenem, ceftaroline	Morrisette et al. 2020 (187)
Burkholderia cepacia	*in vitro* and *in vivo* (*Galleria mellonella*)	KS12, KS14 + ceftazidime/meropenem/ciprofloxacin/levofloxacin/tetracycline/minocycline	Kamal et al. 2015 (188)
Staphylococcus aureus	*in vitro*	SAP-26 + vancomicin/rifampicin/azithromycin	Rahman et al. 2011 (189)
	in vitro	SA5 + gentamicin	Kirby 2012 (190)
	in vitro and *in vivo*	Sb-1 + teicoplanin	Yilmaz et al. 2013 (162)
	in vitro and *in vivo*	MR-10 + linezolid	Chhibber et al. 2013 (191)
	in vitro and in vivo (Galleria mellonella)	SAL200+nafcillin/vancomycin	Kim et al. 2018 (192)
	in vitro	CHAP-amidase* + vancomycin	Kashani et al. 2017 (124)
	in vitro	Sb-1 + vancomycin, daptomycin, fosfomycin, gentamicin, flucloxacillin, cefazolin, rifampin	Wang et al. 2020 (193)
	in vitro	φSZUT, φSZIP1, φSZIP2 + amoxicillin, ampicillin, cefadroxil, ciprofloxacin, chloramphenicol, cefixime, tetracycline	Iqbal et al. 2020 (183)
	in vitro	Sb-1 + daptomycin, vancomycin	Kebriaei et al. 2020 (194)
	in vitro and *in vivo* (rats)	J-Sa36, Sa83, Sa87 + clindamycin, azithromycin, erythromycin	Liu et al. 2021 (195)
	in vitro	Henu2 + tetracycline, cefotaxime, linezolid, clarithromycin, ciprofloxacin	Li et al. 2021 (196)
Streptococcus pneumoniae	*in vitro* and *in vivo* (mouse)	Cpl-711* + cefotaxime/amoxicillin	Letrado et al. 2018 (120)
Mycobacterium smegmatis, M. tuberculosis	*in vitro*	D29/TM4/DS6A, D29/TM4/Che7, PDRPv/PDRxv) + rifampicin, isoniazid	Kalapala et al. 2020 (197)
Salmonella spp.	*in vitro*	φSZUT, φSZIP1, φSZIP2 + amoxicillin, ampicillin, cefadroxil, ciprofloxacin, chloramphenicol, cefixime, tetracycline	Iqbal et al. 2020 (183)

Table 1. Studies with combined phage and antibiotic therapies. Marks (*) references endolysin-related studies.

There have been some clinical experiences using phage therapy and phage therapy combined with antibiotics. In 2011, a 67-year-old woman with failures in urinary tract infection by *P. aeruginosa* treatments with gentamicin, ceftazidime,

meropenem and ciprofloxacin (relapses occurred after 7 days of stopping the treatment) was treated with a personalized pyophage cocktail of six phages with a combination with colistin and meropenem (160). The success of the treatment resulted in sterile urine for 6 months after bacterial treatment. In 2017, a 68-year-old diabetic man developed a disseminated infection from pancreatitis provoked by MDR *A. baumannii* and after 4 months of multiple antibiotic treatments, he was treated with phage cocktails at different periods and minocycline managed to the elimination of *A. baumannii* infection and clinical improvement, including the return of normal life of the patient after a year (155). In 2018, another case of *P. aeruginosa* of a 76-year-old patient with aortic graft infection and without any possibility of surgical intervention was treated with a mixture of phage OMKO1 and ceftazidime (165).4 weeks after the first administration, the infection completely disappeared and he did not have any recurrent infection.

The lung infection, surgical wounds and skin lesions improved with phage therapy and *M. abscessus* was finally not isolated in the patient. Besides in 2019, there have been at least 3 more cases of phage therapy with positive outcomes with phage-antibiotic combinations in CF, lung transplant and osteomyelitis (157, 198, 199). More randomized controlled clinical trials of comparisons between standard antibiotic therapies against phage-antibiotic combination therapies are urgently needed (141).

4. Bacterial-phage interactions

The constant coevolution between bacteria and phage causes counter-adaptation between the interacting species populations, as they are highly dynamic over time (200). The type of interaction depends upon multiple variables, such as the genomic content of the phage, if the infection follows a lytic or lysogenic cycle or even the bacterial supply resources (200, 201). This results in negative effects in the bacteria, such as the development of phage-resistance mechanisms, which has fitness costs for the bacteria and/or higher susceptibility to other phage infections (202, 203); or beneficial effects, such as some prophages conferring a large and diverse number of new functions to the

bacteria (50). For example, they can help the host's genome to regulate gene expression, introduce new functions (e.g. related to virulence, metabolism, antibiotic resistance) or lyse competing bacterial communities (204-207).

4. a. Phage-resistance mechanisms

The global growth of antibiotic resistance has renewed interest in phage therapy where phage cocktails, as well as the combination of phages and antibiotics, have been successful in treating infections by MDR bacteria. To optimize phage therapy, we need to understand how bacteria evolve against phage attack. The appearance of bacterial variants with phage resistance is one of the main problems when is carried the search for the "perfect" phage treatment. One of the alternatives to overcome this problem is to synergistically combine a treatment based on phage with an antibiotic dose. However, when selecting the appropriate phage for therapy, the capacity to develop resistance to this phage must be taken into account. The use of genomics to track antimicrobial resistance is increasingly developed and used in clinical laboratories (208). For that reason, it is important to consider, in an emerging future with phage therapy, to detect and avoid phage resistant strains.

Besides, out of the clinical environment, an effort has been made in the industry to design biotechnologically bacteria, which are continuously exposed to phage attack and considerably harms industrial production, with defence mechanisms against this attack. Understanding which type of mechanisms and how their function works is mandatory to know the best mechanism for a certain type of bacteria.

Due to the coexistence with phages, bacteria have developed various phage defence mechanisms. Bacterial phage resistance emerges through chromosomal mutations, developing a wide range of antiviral mechanisms targeting any phase of the phage life cycle (82). The development of bacterial phage resistance was first described almost a century ago by Luria and Delbrück, who observed that the initial phase of lysis of phage was followed by bacterial regrowth due to the selection of phage resistant subpopulations in a process of coevolution: a process of reciprocal adaptation in which two or more species evolve through selective pressure on one another (209). Later, other

studies demonstrated this coevolution between host bacteria and phages, such as in *E. coli* O157: H7 and phage PP01, where Mizoguchi et al. showed that the bacteria which survived in a culture with phages had alterations in the lipopolysaccharide (LPS) or the inactivated surface protein OmpC (210) In the last years, new bacterial mechanisms to generate or modify phage resistance have been discovered and characterized. Although a large part of defence systems against phages already existed in bacteria, the appearance of phage-resistant organisms is associated with spontaneous mutations and adaptation, especially with modifications in the receptors that phages use to adhere to the cell (82). The main phage resistance mechanisms are related to the inhibition of phage adsorption, blocking of the phage DNA injection, cutting of injected DNA, inhibition of the phage DNA replication, interference in phage assembly, and bacterial suicide (83, 84) (**Figure 4**):

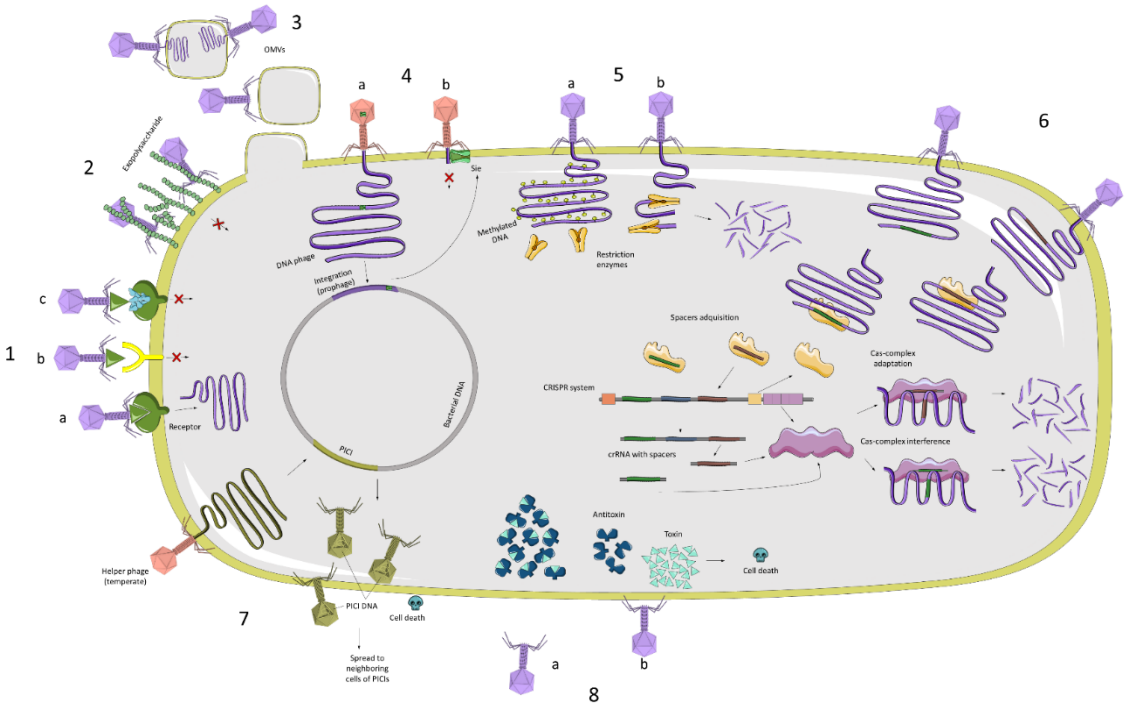

Figure 4. Representation of the main mechanisms of bacterial resistance against phage infection. This figure is also part of the pre-print version of the article in the third chapter of this thesis.

- Inhibition of the adsorption can be developed through different mechanisms, such as the Outer Membrane Vesicles (OMV), which are structures composed of a lipid membrane used as a natural decoy to

defend bacteria against harmful substances (211-213); such as the alteration or blockage of the phage receptor in the bacterial membrane (214, 215); or producing a higher level of extracellular matrix (216).

- Blocking of the DNA injection due to the Superinfection Exclusion (Sie) systems. Sie proteins are mainly found in prophages. They are coded to block the entry of other bacteriophages in the cell by masking the host factors required for DNA phage infection or interacting with a structural element of the phage (217-220).
- DNA cleavage. Once the DNA entry in the host, bacteria have enough defence mechanisms, both innate and adaptative. R-M (restriction-modification) or the R-M-like system DISARM systems are examples of innate immunity to phages (221-223). When unmethylated phage DNA enters bacteria with an R-M system it will be cleaved by the restriction enzyme. The DISARM (Defence Island System Associated with Restriction-Modification) consists of five-gene cassettes which proteins like helicases or methyltransferases. Its mechanism remains to be elucidated. CRISPR-Cas system forms part of the adaptative defence of the bacteria (224, 225). Bacteria with this system integrates small fragments of foreign DNA (spacers) into the CRISPR loci. This spacer sequence leads the Cas nuclease protein to cleave the complementary nucleic acids that enter the cell.
- Replication inhibition through the less unknown BREX (BacteRiophage EXclusion) system, which is known to allow the adsorption but not the replication of the phage (226, 227). BREX is a recently discovered mechanism similar to the R-M systems preventing the phage replication but differs as DNA cleavage was undetectable. However, the mechanism by which BREX prevents infection is yet to discover.
- Assembly interference. PICI (Phage-Inducible Chromosomal Island) can excise from the bacterial DNA and assembly itself to produce phage particles with its own PICI-DNA (228). PICIs are a newly discovered and ubiquitous type of mobile genetic element with a wide range of effects on bacterial pathogenicity. In *E. coli*, PICIs are able to interact with the terminase small subunit of the phages, forming a heterocomplex unable

to recognize the phage DNA thereby blocking the phage packaging but promoting the packaging of the PICI elements (229).

- Bacterial suicide. The last bacterial defence mechanism results in bacterial death. Abortive infection or Abi systems can interrupt the bacterial development in any phase after the DNA entrance (replication, transcription or translation) resulting in less phage liberation and thus protecting the population against a general infection. They are common in mobile genetic elements such as plasmids or prophages (230, 231). One of the most extended Abi systems is the Toxin-Antitoxin (TA) system (232, 233).

4. b. CRISPR-Cas system in bacteria

CRISPR-Cas systems (clustered, regularly interspaced short palindromic repeats-CRISPR-associated proteins) are RNA-guided adaptative immunity systems that exist in bacteria (~40%) and archaea (~90%). They prevent the host to be infected by phages, viruses and from being harmed by other foreign genetic elements (234, 235). The first observations of the CRISPR repeats were made by Ishino et al. in 1987 (236), but without developing any biological explanation about these sequences. Not until 1993 a Spanish microbiologist, Francisco Mojica, gave these sequences the name CRISPR (237).

The CRISPR loci consist of several direct repeats separated by variable sequences. These variable sequences are called spacers and they correspond to captured viral and plasmid DNA sequences. This loci is often adjacent to the CRISPR-associated proteins or *cas*: a large and heterogeneous group of proteins with functional domains such as nucleases, helicases, polymerases and/or DNA/RNA-binding proteins (238).

4. c. Relationship between phages and quorum sensing

Quorum Sensing (QS) was first described in 1970 by Nealson *et al*. in *Vibrio fischeri* (239). However, the concept “quorum sensing” was first quoted in 1994 by Fuqua *et al.* about a mechanism by which bacteria have cooperative patterns to explain certain behaviours (240). QS is a regulatory mechanism of the genetic expression in bacteria in response to cellular density (241). It has been identified and characterized in gram-positive and gram-negative bacteria. In

gram-positive bacteria, the autoinducer signal is an oligopeptide that is detected by two different mechanisms (242). In gram-negative bacteria, the autoinducers are acyl-homoserine lactones (AHLs) and quinolones, which bounds to specific receptors in the inner membrane or the cytoplasm and then these receptors modify a wide variety of genes in various biological processes. The activation of QS also stimulates the synthesis of an autoinducer in the bacteria establishing feedback in the bacterial population (243).

QS have been proved to be a therapeutic target of interest in multidrug-resistant microorganisms. For example, QS has a crucial role in the formation of biofilm (244). The blockage of specific QS signals could prevent biofilm formation in many pathogens, thus increasing the sensitivity of pathogens to antimicrobial agents and improving the effectiveness of antibiotics (245). Furthermore, QS has been proved to regulate virulence factors crucial for the pathogenesis of infections, which can evade the immune response of the host and cause pathological damage (246). In *P. aeruginosa*, QS is able to control pyocyanin production, elastase, lectin, exotoxin A and other virulence factors (247). The inhibition of the QS, also called Quorum Quenching (QQ), is considered a potential therapeutic alternative (248). The autoinducer compounds can be enzymatically degraded through the QQ mechanism, blocking its production and its reception through inhibition (249). The QQ enzymes are being studied as potential quenchers of QS to prevent microbial infection (250), for example, the characterization of the expression network in *A. baumannii* clinical strains due to the regulation of the AidA protein (251).

The relation between QS and the bacteriophage infection has been analysed by several authors, thus, the phage φpa3 has been proved to transduce mutations in QS genes in *P. aeruginosa* PAO1 (252). Also, it was demonstrated that QS systems can reduce the phage receptor numbers at the stationary phase, acting as a defence mechanism in bacteriophage infection in *E. coli* (253), and vary the expression of the receptor OmpK for *Vibrio anguillarum* to control the infection of the phage KVP40, reducing receptor expression under conditions of high infection risk (254). In *V. cholerae*, QS protects against attack by lytic bacteriophages such as JSF35 (255). Also, in *V. cholerae*, QS was demonstrated to control the change from a lysogenic cycle to a lytic one in the

vibrio phage VP882 by QS-related genes encoded by the bacteriophage itself (256). Finally, bacteriophages have been found to select bacterial cells with functional QS systems in isolates of *P. aeruginosa* (257) as well as in clinical strains of *A. baumannii* prophages AbAb105-1ϕ and Ab105-2ϕ select this QS functional strains (14). The recent increase in the sequencing of whole bacterial genomes has shown a large number of combined prophage and pathogenic strains seem to have a high proportion of phage-related genes than non-pathogenic strains (258-260).

OBJECTIVES

The multi-drug resistant (MDR) pathogens are responsible for most nosocomial infections. Phage therapy is a promising strategy against MDR bacteria. The actual next-generation sequence techniques are employed to identify new prophages and their undesirable genes, like toxins or virulence genes, and those useful genes in antimicrobial therapy, as endolysins. These techniques combined with the DNA recombinant techniques can provide novel resources in phage therapy, as the mutant lytic phages derived from lysogenic phages and the endolysins identified and purified from these lysogenic phages. The combination of phage therapy with antibiotic therapy may improve the effectiveness of phage therapies. Furthermore, it is important to reveal the role and functions in which lysogenic phages participate and vice versa, such as QS.

The objectives of this doctoral research are:

Chapter I

Transform the lysogenic phage Ab105-2phi from *A. baumannii* into a lytic one with potential use in phage therapy.
Reduce the phage resistance emergence by combining the mutant lytic phage with carbapenem antibiotics as a potential treatment against *A. baumannii* clinical strains.

Chapter II

To identify and characterize new potential endolysins, from *A. baumannii* prophages, with antimicrobial activity against gram-negative members of the *ESKAPE* group.
To combine the endolysins obtained with colistin, to increase the access of the endolysin to the peptidoglycan and increase the antimicrobial activity.

Chapter III

To show *in silico* the phage resistance genes presence and CRISPR arrays present in 18 genomes of *A. baumannii* clinical strains from the 2000 and 2010 years and its evolution through the years.

Chapter IV

To elucidate the relationship of the prophages and clinical strains of *P. aeruginosa* from CF patients.

To discover phages potentially involved in mechanisms such as virulence, resistance or QS in CF clinical isolates of *P. aeruginosa*.

CHAPTERS

Chapter I. Combined Use of the Ab105-2φΔCI Lytic Mutant Phage and Different Antibiotics in Clinical Isolates of Multi-Resistant *Acinetobacter baumannii*

The need for the development of new treatments against MDR has led to a renewed interest in bacteriophage therapy, abandoned in the western world when antibiotics emerged in the 1920 decade but maintained in eastern countries such as USSR and Poland (44).

Bacteriophages are good candidates as antimicrobial agents, as they have a narrow spectrum of activity protecting the normal microbiota, they multiply in the infection site, are abundant in nature and easily isolated and their production has low costs. The combination of phages with antibiotics has been demonstrated to be synergic in several studies, and also can reduce the emergence of antibiotic and/or phage resistance (142, 261).

In this chapter, we have developed a strategy that can expand the availability of phages useful in phage therapy, by obtaining a lytic mutant phage from a lysogenic one. Moreover, its antimicrobial activity was characterized alone and in combination with antibiotics.

The lysogenic phage Ab105-2phi (Gb: KT5880759), identified in the genome of the *A. baumannii* clinical strain Ab105_GEIH-2010 (14), was transformed into a therapy secure lytic phage, by deleting the CI repressor gene, which regulates the maintenance of the lysogenic state of the phages (56)

The resultant bacteriophage, Ab105-2phiΔCI, was isolated and observed by Transmission Electron Microscopy (TEM) showing the same Siphoviridae structure as the wild type phage. The host range covered 25% of the strains tested and the Efficiency of Plating (EOP) was the highest in the strain Ab177_GEIH-2000. For this reason and for not having complete prophages (14), this strain was selected for the following assays. The adsorption time was 12 minutes and the one-step growth curve revealed a latent period of 30 min and a burst size of around 32 ± 2 PFU per infected cell. The antimicrobial activity against biofilm of the phage against biofilm showed a significant reduction in biofilm biomass.

The infection curves obtained with the original lysogenic phage, Ab105-2phi and with the mutant lytic phage Ab105-2phiΔCI confirmed the lytic nature of the mutated phage, as the growth of the infected culture decreased drastically but regrowth was observed at 5 hours due to the emergence of phage resistant mutants.

The rate of phage resistant mutants was established, and a reduction of almost 1 log in the CFU counts of the host strain, was observed when the infection was done with the mutant lytic phage Ab105-2phiΔCI in combination with the antibiotics meropenem, imipenem and doxycycline, instead of when the mutant lytic phage was alone.

We developed a bacterial killing assay in presence of a combination of the lytic mutant phage Ab105-2phiΔCI at three different Multiplicity of Infection (MOIs: 0.1, 1 and 10) and three antibiotics (doxycycline, meropenem and imipenem) at two different Minimum Inhibitory Concentrations (MICs: 1/4 and 1/8). No synergistic effects were observed with doxycycline, except for a slight decrease when the combinations include phage concentration at MOI 10. In an opposite way, a reduction in the number of CFUs was detected after 6 h for all combinations of meropenem and imipenem with the phage (between 4 and 8 log of difference of CFU/mL), demonstrating a synergistic effect. This synergistic outcome was held after 24 h when the concentration of carbapenemic antibiotics was at 1/4 MIC in the combination of a MOI 10 of phage in the case of meropenem and MOI 1 and MOI 10 in the case of imipenem, with a reduction of around 6 log CFU/mL per each case.

Finally, in the *Galleria mellonella* survival assay the survival rate was higher when the larvae infected with the Ab177_GEIH-2000 strain was treated with the combination of meropenem and imipenem plus the mutant lytic phage Ab105-2phiΔCI, but being only statistically significant ($p < 0.05$) with the imipenem combination.

The corresponding paper at *MDPI Microorganisms* journal is attached:

microorganisms

MDPI

Article

Combined Use of the Ab105-2φΔCI Lytic Mutant Phage and Different Antibiotics in Clinical Isolates of Multi-Resistant *Acinetobacter baumannii*

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Abstract: Phage therapy is an abandoned antimicrobial therapy that has been resumed in recent years. In this study, we mutated a lysogenic phage from *Acinetobacter baumannii* into a lytic phage (Ab105-2phiΔCI) that displayed antimicrobial activity against *A. baumannii* clinical strain Ab177_GEIH-2000 (isolated in the GEIH-REIPI Spanish Multicenter *A. baumannii* Study II 2000/2010, Umbrella Genbank Bioproject PRJNA422585, and for which meropenem and imipenem MICs of respectively, 32 μg/mL, and 16 μg/mL were obtained). We observed an in vitro synergistic antimicrobial effect (reduction of 4 log–7 log CFU/mL) between meropenem and the lytic phage in all combinations analyzed (Ab105-2phiΔCI mutant at 0.1, 1 and 10 MOI and meropenem at 1/4 and 1/8 MIC). Moreover, bacterial growth was reduced by 8 log CFU/mL for the combination of imipenem at 1/4 MIC plus lytic phage (Ab105-2phiΔCI mutant) and by 4 log CFU/mL for the combination of imipenem at 1/8 MIC plus lytic phage (Ab105-2phiΔCI mutant) at both MOI 1 and 10. These results were confirmed in an in vivo model (*G. mellonella*), and the combination of imipenem and mutant Ab105-2phiΔCI was most effective ($p < 0.05$). This approach could help to reduce the emergence of phage resistant bacteria and restore sensitivity to antibiotics used to combat multi-resistant strains of *Acinetobacter baumannii*.

Keywords: *Acinetobacter baumannii*; multiresistant; mutant lytic phage; phage therapy; antibiotic-phage synergy

1. Introduction

Multi-drug resistant (MDR) bacteria, such as *A. baumannii* are considered to be a major concern by the World Health Organization (WHO), because of their ability to acquire antimicrobial resistance via intrinsic characteristics and mechanisms (e.g., presence of the outer membrane) or via mechanisms acquired by horizontal genetic transfer [1,2]. This situation has led to an urgent need to develop new antimicrobial agents and to a renewed interest in phage therapy. Phage therapy was first developed in the 1920s but was abandoned in the Western world after the discovery of antibiotics. However, the use of phage therapy continued in Eastern countries, such as Poland and USSR, where bacteriophages are used for the prophylaxis and treatment of infections, such as dysentery, ulcers, and methicillin resistant *Staphylococcus aureus* (MRSA) infections [3,4].

Phage therapy is now considered a real option for treating MDR bacteria, and there are some examples of its use in treating human patients [5]. Phages are bacterial viruses, and like other viruses, they are obligate parasites that enter host cells through mechanisms that are based on receptor recognition. Genetic material is then injected into the bacteria and use the bacterial machinery to produce phage proteins [6,7]. Phages generally undergo a lytic (virulent) or lysogenic (temperate) life cycle. Lytic phages infect, and rapidly lyse and kill host cells, releasing phage progeny into the surrounding medium. Lysogenic phages infect the host cell and integrate their nucleic acid into the host genome, or exist as plasmids in the host cells, remaining in a stable prophage state for generations. Prophages can be "induced" to exit the cell as lytic phages under some conditions, such as the presence of antibiotics [8,9]. The lysogenic/lytic cycle of temperate bacteriophages is controlled by Cro, CI, and CII proteins; the Cro protein induces the lytic state and the CI repressor protein inhibits the Cro protein, thereby inducing the lysogenic state [10].

Only lytic phages are used in phage therapy as lysogenic phages can transfer resistance genes or virulence factors to the host [11].

The combined use of antibiotics and phages has been tested in several studies, demonstrating strong control of the bacteria, and a reduction in the development of phage and/or antibiotic resistance [12,13]. Phages are good candidates for use in combination with antibiotics for various reasons, including that they have a different mechanism of action from antibiotics; hold a narrow spectrum of activity, which protects the normal microbiota; they can multiply at the infection site; they are abundant in nature and can be easily isolated; and production costs are low [14–16].

In this study, we produced a mutant lytic phage from a lysogenic phage, that is incorporated in the genome of a clinical strain of *A. baumannii* by deleting the CI repressor gene, and thus, preventing the entry of the phage into the lysogenic cycle [10,17]. We then tested the antimicrobial activity of the novel lytic phage, Ab105-2phiΔCI, in combination with carbapenem antibiotics (meropenem and imipenem) against a carbapenem-resistant strain of *A. baumannii*. The combined therapy enhanced the antimicrobial activity of both, the phage and the antibiotic; the bacterium became sensitive to the antibiotics and the emergence rate of phage resistant bacteria was reduced.

2. Material and Methods

2.1. Bacterial Strains

In this study, we used 20 clinical strains isolated from Spanish hospitals during the GEIH-REIPI Spanish Multicenter *Acinetobacter baumannii* Study II 2000–2010, GenBank Umbrella project PRJNA422585 (https://www.ncbi.nlm.nih.gov/bioproject) (Table 1).

Table 1. Bacterial strains used in this study. Phage host range determined by spot test and efficiency of plating (EOP).

Strain	ST	Spot	EOP	Spanish Hospital Where the Strain Was Isolated
Ab105_GEIH-2010	2	+/−	1	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab192_GEIH-2000	2	+/−	0.22	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab404_GEIH-2010	80	+	0.0002	Hospital Dr. Molines (Valencia, Spain)
Ab166_GEIH-2000	2	+/−	-	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab177_GEIH-2000	2	+	1.55	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab13_GEIH-2010	79	-	-	Hospital Santiago de Compostela (Santiago de Compostela, Spain)
Ab09_GEIH-2010	297	-	-	Hospital Santiago de Compostela (Santiago de Compostela, Spain)
Ab160_GEIH-2000	2	-	-	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab155_GEIH-2000	2	-	-	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab05_GEIH-2010	186	-	-	Hospital A Coruña (A Coruña, Spain)
Ab22_GEIH-2010	52	-	-	Hospital Pontevedra (Pontevedra, Spain)
Ab421_GEIH-2010	2	-	-	Hospital Insular (Gran Canaria, Spain)
Ab77_GEIH-2000	2	-	-	Hospital Universitario Ramon y Cajal (Madrid, Spain)
Ab141_GEIH-2000	179	-	-	Complejo Hospitalario Toledo (Toledo, Spain)
Ab217_GEIH-2010	2	-	-	Hospital Reina Sofía (Cordoba, Spain)
Ab235_GEIH-2010	2	-	-	Hospital Marqués de Valdecilla (Santander, Spain
Ab37_GEIH-2010	2	-	-	Hospital Virgen del Rocío (Seville, Spain)
Ab222_GEIH-2000	181	-	-	Hospital Bellvitge (Barcelona)
Ab461_GEIH-2010	2	-	-	Hospital del Mar (Barcelona, Spain)
Ab173_GEIH-2010	88	-	-	Hospital San Agustín (Avilés, Spain)

ST: Sequence Type. Spot test: (+) clear spot; (+/−) turbid spot; (-) no spot.

2.2. Obtaining the Lytic Phage Mutant

The bacteriophage sequence Ab105-2phi (Genbank: KT5880759) detected in clinical strain *A. baumannii* Ab105GEIH_2010 was analyzed and the CI gene identified as ORF 17. The CI gene was deleted by double homologous recombination with the suicide vector pMo130TelR [18,19]. The primers were first designed for the amplification of the flanking regions (1000 bp) of the CI gene. These regions were amplified by PCR and ligated and cloned into the pMo130telR vector (Table 2). This construction was transformed in *Escherichia coli* DH5α to produce large numbers of the plasmid with the gene flanking regions. The plasmid was purified and transformed in the *A. baumannii* Ab105 clinical strain by electroporation, and incubated for two hours at 37 °C without antibiotic, thereby producing a recombinant wild type with the mutated gene integrated in its genome. Finally, the mutants were selected in the presence of kanamycin (50 μg/mL). In order to isolate only those mutants with the CI gene deletion in the chromosome, the plasmid loss was induced in the absence of kanamycin, and the recombinant clones were selected in the presence of 15% sucrose. In order to isolate the mutant phage Ab105-2phiΔCI from the bacterial clones by which the CI gene was deleted, a selected clone was incubated in LB broth, which is supplemented with mitomycin (10 ug/mL) to induce release of the phages. The supernatant was collected, treated with chloroform, and filtered (20 μm). The filtered supernatant was used to infect the clone without the phage, and plaques were obtained by the agar

overlay method [20]. A clear plaque was isolated by PCR and sequencing was conducted to confirm the correct deletion of the CI gene.

Table 2. Primers used to delete the CI gene.

Primer	Sequence	Strain/Plasmid
UPCI [NotI]Fw	GGG*GCGGCCGC*TGAAGAATTCATCACTTG	Ab105_GEIH-2010
UPCI[BamHI]Rev	GGG*GGATCC*CGTTACTTCTATCGGAAT	Ab105_GEIH-2010
DWCI[BamHI]Fw	GGG*GGATCC*ATTAAGGTTTTAGGTGAT	Ab105_GEIH-2010
DWCI[SphI]Rev	GGG*GCATGC*TAAATCATCCAAATCGAC	Ab105_GEIH-2010
CIFw	ATGGACAAATTTATGGCTAC	Ab105_GEIH-2010
CIRev	TAACTTTTTCTAACACGCT	Ab105_GEIH-2010
IntCIFw	AAAGCGCTGCCAACTTTT	Ab105_GEIH-2010
IntCIRev	CAACAGATTCATCCTCAT	Ab105_GEIH-2010
pMo130TelRFw	ATTCATGACCGTGCTGAC	pMo130TelR
pMo130TelRRev	CTTGTCTGTAAGCGGATG	pMo130TelR
Plasmid	**Description**	**Origin**
pMo130TelR	Suicide plasmid, *xylE*$^{+}$, *sac*B^{+}, km^{R}, Tel^{R}	[19]

Restriction enzyme sites are shown in italics.

A clone of strain Ab105GEIH_2010, induced with mitomycin, was isolated and excision of the phage was confirmed by PCR of the CI gene and the flanking regions (1000 pb each region) of the gene.

2.3. Host Range and Efficiency of Plating Analysis

The host range of the lytic mutant phage Ab105-2phiΔCI was established by applying the spot test [21] to the 20 clinical strains of *A. baumannii* under study. Efficiency of Plating (EOP) was established as the ratio between the test strain titre and the host strain titre [22].

2.4. Transmission Electron Mmicroscopy (TEM) and Live-Cell Imaging

A broth culture of strain Ab177_GEIH-2000 was infected with the lytic mutant phage Ab105-2phiΔCI. The lysates were centrifuged at 3400× *g* for 10 min and the supernatant was filtered through a 0.22 μm filter (Merck Millipore, Ltd. Tullagreen, Carrigtwahill, Co Cork, Ireland). NaCl was added to a final concentration of 0.5 M, and the suspensions were mixed thoroughly and left on ice for 1 h. The suspensions were centrifuged at 3400× *g* for 40 min at 4 °C, and the supernatants were transferred to sterile tubes. PEG 6000 (10% *w/v*) was added, dissolved, and incubated overnight at 4 °C. Bacteriophages were then precipitated at 3400× *g* for 40 min at 4 °C and resuspended in SM buffer (0.1 M NaCl, 1 mM MgSO4, 0.2 M Tris-HCl, pH 7.5) [23]. The samples were negatively stained with 1% aqueous uranyl acetate before examination by electron microscopy.

Live-cell imaging was carried out by time-lapse microscopy after initial adsorption of the mutant lytic phage Ab105-2phiΔCI to the clinical strain Ab177_GEIH-2000 at 37 °C in agar slices, which were placed directly between stainless steel O-rings. The use of extracellular DNA markers enabled the lysis of more than 300 bacteria to be monitored in real time.

2.5. Adsorption Curve, One Step Growth Curve, and Infection Curve

An overnight culture of *A. baumannii* clinical strain Ab177_GEIH-2000 was diluted 1:100 in LB broth, and incubated at 37 °C at 180 rpm, until an early logarithmic phase, i.e., at an optical density of 0.2 (OD 600nm). At this point the culture was infected with the lytic mutant phage Ab105-2phiΔCI at

a multiplicity of infection (MOI) of 0.1. The adsorption curve and the one step growth curve were determined after growing the phage in LB, supplemented with $CaCl_2$, as previously described [20,24]. In the one step growth curve, the latent period was defined as the interval between adsorption of the phages to the bacterial cells and the release of phage progeny. The burst size of the phage was determined as the ratio of the final number of free phage particles to the number of infected bacterial cells during the latent period [22].

An early exponential culture of the strain Ab177_GEIH-2000 in LB, supplemented with $CaCl_2$, was infected with the lysogenic phage Ab105phi2 and the mutant lytic phage Ab105phi2ΔCI at different MOIs (0.1, 1 and 10), and the corresponding infection curves were constructed. The phage cultures were maintained at room temperature during the adsorption period and then incubated at 37 °C and 180 rpm for 6 h. The optical density was measured at intervals of one hour during this period.

2.6. Frequency of Occurrence of Phage Resistant Bacteria

Phage resistant mutants were produced as previously described [25]. To determine the emergence of phage resistant mutants, an overnight culture of strain Ab177_GEIH-2000 was diluted 1:100 in LB and grown to an OD600nm of 0.6–0.7. An aliquot of 100 µL of the culture containing 10^8 colony forming units (CFU)/mL was serially diluted, and each dilution mixed with 100 µL of 10^9 plaque forming units (PFU)/mL, and plated by the agar overlay method [20]. The plates were incubated at 37 °C for 18h and the number of CFUs was counted. The same procedure was used to produce phage resistant mutants in the presence of the antibiotics doxycycline, meropenem, or imipenem, which were added to the plates, each at 25% of the minimum inhibitory concentration (MIC). The frequency of occurrence of phage resistant mutants and phage-antibiotic resistant mutants was calculated by dividing the number of resistant bacteria by the total number of sensitive bacteria.

2.7. Antimicrobial Activity of the Mutant Lytic Phage Ab105-2phiΔCI in Biofilm

An overnight culture of the *A. baumannii* clinical strain Ab177_GEIH-2000 was diluted 1:100 and used to inoculate 100 µL of LB in some wells of a 96 multi-well plate. The plate was maintained at 37 °C in static conditions for 4 h. The medium was then discarded and the wells were washed twice with PBS before 100 µL of fresh LB was added. After 24 h at 37 °C, the medium was again discarded and the wells were washed with PBS, and filled with 90 µL of SM buffer, then 10 µL of phage Ab105-2phiΔCI (10^7 PFU/mL) was added. SM buffer (100 µL) was added to control wells. The plates were then incubated at 37 °C for 24 h. Finally, the supernatant was discarded and the wells were washed with PBS. Half of the wells were used to quantify the CFUs and the other half were used to quantify the biofilm. PBS (100 µL) was added to the wells used to quantify the CFUs and the plates were agitated for 5 min and sonicated for another 5 min. The suspension was serially diluted and plated on LB plates. For quantification of the biofilm, 100 µL of methanol was added to each well and discarded after 10 min. Once the methanol had completely evaporated, 100 µL of crystal violet (0.1%) was added and discarded after 15 min. Finally, the wells were washed with PBS before the addition of 150 µL of acetic acid (30%), and the absorbance was measured at OD 595 nm.

2.8. Antimicrobial Activity in Combination with Antibiotics

A bacterial killing assay was constructed to determine the synergy of phage Ab105-2phiΔCI in combination with meropenem, imipenem and doxycycline at 1/8 and 1/4 of the respective MICs (meropenem 32 µg/mL, imipenem 16 µg/mL and doxycycline 64 µg/mL). An overnight culture of the tested strain was diluted at 1:100 in LB broth supplemented with 10uM $CaCl_2$ and incubated at 37 °C and 180 rpm until the culture reached an early exponential phase at 0.2 OD (600nm). At this point, antibiotic and the Ab105-2phiΔCI phage were added to the culture. The flasks were maintained at room temperature during the adsorption period before being incubated at 37 °C and 180 rpm for 24 h. Aliquots were removed after 6 h and 24 h and were serially diluted and plated in LB plates for subsequent counting of CFU.

2.9. Galleria mellonella Survival Assay

The *Galleria mellonella* model used was an adapted version of a previously developed model also used to study bacteriophage therapy [26,27]. The procedure was as follows: twelve *G. mellonella* larvae, acquired from TruLarvTM (Biosystems Technology, Exeter, Devon, UK), were each injected in the left proleg with 10 µL of a suspension of *A. baumannii* Ab177_GEIH-2000, diluted in sterile phosphate buffer saline (PBS) containing 1×10^5 CFU (± 0. 5 log). The injection was performed with a Hamilton syringe (volume 100 µL) (Hamilton, Shanghai, China). One hour after infection, the larvae were injected in the right proleg with 10 µL of the lytic mutant phage Ab105-2phiΔCI, at MOI 10, in combination with meropenem at 1/4 MIC and imipenem at $\frac{1}{4}$ MIC. The controls included 10 µL of the lytic mutant phage Ab105-2phiΔCI at MOI 10, or meropenem at 1/4 MIC and imipenem at 1/4 MIC. The injected larvae were placed in Petri dishes and incubated in darkness at 37 °C. The number of dead larvae was recorded after 72 h. The larvae were considered dead when they showed no movement in response to touch [26].

The survival curves for the in vivo *G. mellonella* infection model were constructed using GraphPad Prism v.6 (San Diego, CA, USA), where the data were analyzed using the Log-Rank (Mantel-Cox, City, State if USA, Country) test. The data were expressed as mean values, and the differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Obtaining the Lytic Mutant of the Phage Ab105phi-2ΔCI

After deleting the CI gene from the temperate phage Ab105-phi2, as previously reported in *Salmonella* [17], we obtained a lytic mutant, designated Ab105-phi2ΔCI, which produced characteristic clear lytic plaques. This is in contrast with the turbid plaques produced by the temperate Ab105-phi2 phage (Figure 1A1). PCR of the DNA, isolated from the Ab105-2phiΔCI phage, confirmed the deletion of the CI gene. PCRs were conducted with the CI genes and combinations of these primers with those of the flanking regions, confirming that no amplification was obtained. PCRs with the primers (UPCI[NotI]Fw/DWCI[SphI]Rev) of the flanking regions of the gene CI were also conducted, and the expected region of 2000 pb was obtained (size without the CI gene). Finally, this amplicon was sequenced and the CI gene deletion was confirmed. Excision of the phage was also confirmed in a clone induced with mitomycin, as no positive PCR were obtained with the CI primers or with the flanking region primers.

Infection curves for the temperate phage Ab105-2phi and the lytic mutant phage Ab105-2phiΔCI were constructed and compared, showing that the lytic mutant killed the culture at all MOI levels tested, as reflected by a large decrease in the optical density. Although, a reduction in growth was observed when the culture was infected with the lysogenic phage Ab105-2phi, the decrease was less than with the lytic mutant. In both cases, the reduction in growth was first observed at MOI 10, but regrowth was also first observed at this MOI, probably due to the emergence of resistance (Figure 1B).

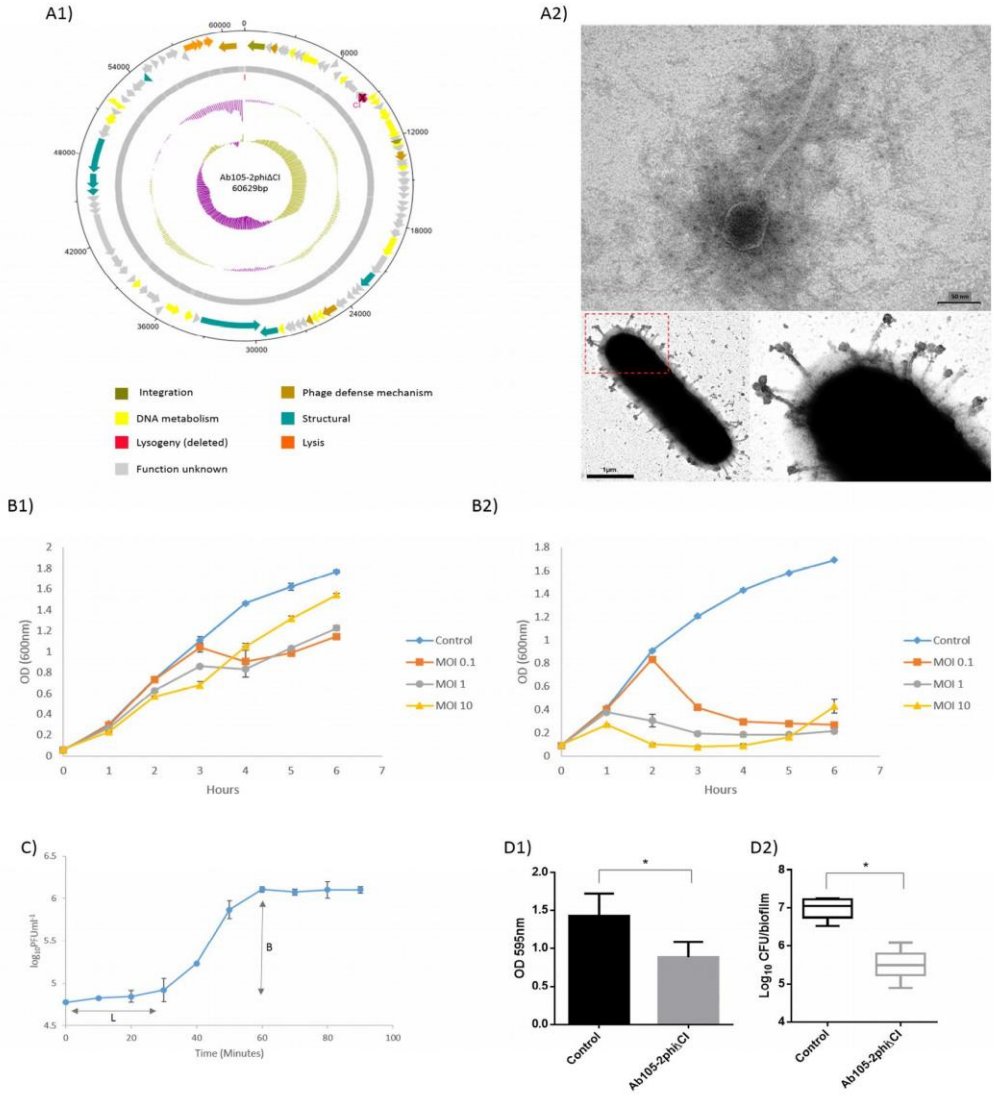

Figure 1. Graphical representation of the Ab105-2phiΔCI phage. The ORF and direction of transcription are indicated by arrows. (**A1**) The protein functions are indicated in different colours, and the GC content and GC skew are shown as pink and green circles respectively. (**A2**) TEM image of the mutant lytic phage Ab105-phi2ΔCI and mutant lytic phage Ab105-phi2ΔCI attached to the cell surface. (**B1**) Infection curves for the lysogenic phage Ab105-2phi and (**B2**) the mutant lytic phage Ab105-2phiΔCI. (**C**) One step growth curve of the mutant lytic phage Ab105-phi2ΔCI (L: Latent period; B: burst size). Mutant lytic phage Ab105-phi2ΔCI antibiofilm activity on the biofilm produced by the clinical strain of *A. baumannii* Ab177_GEIH-2000. (**D1**) Reduction in the biofilm and reduction in the number of CFUs present in the biofilm after treatment with the mutant lytic phage Ab105-phi2ΔCI (**D2**). Figures B, C and D show the mean values +/− SD from three independent assays. Statistically significant differences ($p < 0.05$) were determined by t-Student test (GraphPad Prism v.6).

3.2. Morphology and Host Range of the Lytic Mutant Phage Ab105-phi2ΔCI

The lytic mutant Ab105-2phiΔCI was isolated and the virion morphology was observed by TEM, revealing that this phage has the typical structure of the Siphoviridae as the wild type phage Ab105-phi2 [28]. All plaques were transparent and about 1mm in diameter (Figure 1A2).

The lytic spectrum of activity of the mutant phage Ab105-2phiΔCI covered 25% of the clinical strains of *A. baumannii* tested. The strain Ab177_GEIH-2000 yielded the highest EOP (1.55) (Table 1). This strain was thus selected for further assays.

3.3. Adsorption and One Step Growth Curve

Both, the adsorption and the one step growth curve were established using host strain Ab177_GEIH-2000, as the EOP of this strain was the most appropriate and also because this strain does not have complete prophages, as previously determined [28]. The adsorption time (12 min) was determined in order to establish the one step growth curve, which revealed a latent period of 30 min and a burst size of approximately 32 ± 2 PFU per infected cell (Figure 1C).

3.4. Antimicrobial Activity of the Mutant Lytic Phage Ab105-2phiΔCI on Biofilm

Biofilm was produced with the clinical strain of *A. baumannii* Ab177_GEIH-2000 susceptible to the mutant lytic phage Ab105-phi2ΔCI. The treatment of the biofilm with 10^7 PFU of this lytic mutant phage caused a statistically significant reduction in the biofilm biomass. The antimicrobial activity against the biofilm forming bacteria was confirmed by a decrease in the CFU, quantified in the presence of the mutant lytic phage (Figure 1D).

Finally, the lytic activity of the mutant phage can be observed in Video 1 (Supplementary Materials).

3.5. Determination of the Emergence Rate of Phage Resistant Mutants

Strain Ab177_GEIH-2000 was resistant to meropenem, imipenem and doxycycline (MICs: Meropenem 32 μg/mL, imipenem 16 μg/mL, and doxycycline 64 μg/mL). In all cases the combination of the phage and antibiotic reduced the rate of emergence of phage-resistant mutants, relative to the rate of resistant mutants in the presence of the phage alone (Table 3).

Table 3. Frequency of phage resistant mutants. Phage resistant mutant frequency in the presence of the combination of doxycycline, meropenem and imipenem at $\frac{1}{4}$ MIC in combination with lytic mutant phage Ab105-2phiΔCI (MOI 10) was calculated.

Sample	Frequency of Phage Resistant Mutants
Ab105-2phiΔCI	1.70×10^{-6}
Ab105-2phiΔCI + Doxycycline	1.31×10^{-7}
Ab105-2phiΔCI + Meropenem	2.10×10^{-7}
Ab105-2phiΔCI + Imipenem	1.90×10^{-7}

3.6. Effect of the Combination of Phage and Antibiotic on the Bacterial Killing Assays

Bacterial killing assays were constructed for *A. baumannii* clinical strain Ab177_GEIH-2000 in the presence of a combination of the lytic mutant phage Ab105-2phiΔCI at different MOIs (0.1, 1, and 10) and three antibiotics (at 1/4 and 1/8 MIC) to which Ab177_GEIH-2000 is resistant: Meropenem, imipenem, and doxycycline (Figure 2).

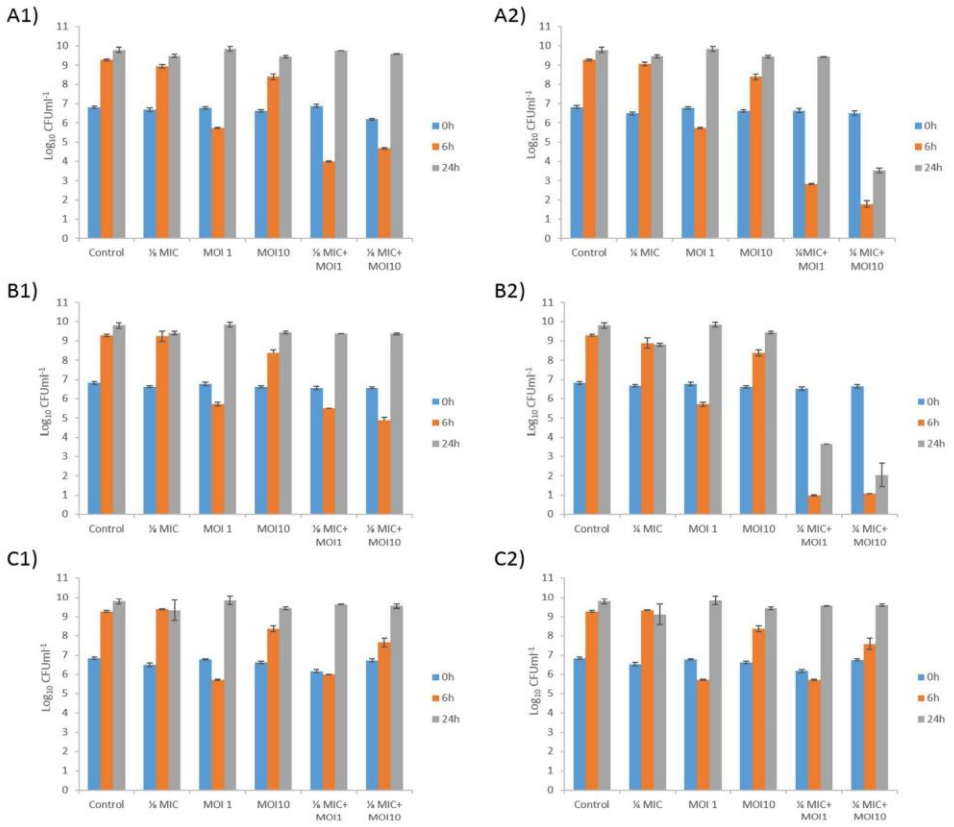

Figure 2. Bacterial killing assays for *A. baumannii* clinical strain Ab177_GEIH-2000 determined using the mutant lytic phage Ab105-2phiΔCI at MOI 1 and MOI10 in combination with meropenem at (**A1**) 1/8 MIC and (**A2**) 1/4 MIC;(**B1**) imipenem at 1/8 MIC and (**B2**)1/4 MIC, and (**C1**) doxycycline at 1/8 MIC and (**C2**)1/4 MIC. Values shown in the graphs are the means +/− SD from three independent assays.

A reduction in the number of CFU was observed with the phage at both MOI 1 (4 log) and MOI 10 (1 log) after 6 h, but no differences from the control were observed after 24 h. The reduction was even greater when the phage was combined with meropenem or imipenem (both carbapenems).

For meropenem plus phage, a synergistic effect was observed after 6 h for all combinations (from 4 log to 7 log CFU/mL). The growth of the *A. baumanni* strain was similar to control levels after 24 h for all concentrations of meropenem plus phage at MOI 1. The synergistic effect was only maintained with the combination of meropenem at 1/4 MIC and phage Ab105-2phiΔCI at MOI10, yielding a difference in bacterial growth of 6 log CFU/mL, relative to that corresponding to the meropenem control (Figure 2A1,2A2).

As with meropenem, the combination of different concentrations of imipenem and the lytic mutant phage had a synergistic effect after 6 h in all cases, with a reduction in bacterial growth of 8 log CFU/mL for the combination of imipenem at 1/4 MIC, plus phage, and 4 log CFU/mL for the combination of imipenem at 1/8 MIC plus phage. The synergistic effect was maintained for 24 h in the combinations of imipenen at 1/4 MIC, with phage at MOI1 and MOI10, but not in the combinations of imipenem at 1/8 MIC and both phage concentrations (Figure 2B1,2B2).

No synergistic effects were observed with doxycycline, and the combination had no more effect than the phage alone at MOI 1. However, when the combinations included the phage at MOI 10,

a slight decrease in the CFU count was observed (less than 1 log CFU/mL), independently of the antibiotic concentration (Figure 2C1,2C2).

The curves obtained for the lytic mutant phage controls showed that Ab177_GEIH-2000 grew at control rates after 24h, due to the acquisition of phage resistance. However, the growth was higher at MOI 10, than at MOI 1 after 6 h, probably because resistance emerges faster at this MOI than at lower MOI.

3.7. Galleria mellonella Survival Assays in the Presence of Meropenem and Imipenem in Combination with the Lytic Mutant Phage Ab105-phi2ΔCI

The combinations of antibiotic and the phage Ab105-2phiΔCI, that resulted in the reduction of the CFU of Ab177_GEIH-2000 at 24h in vitro were assayed in a *G. mellonella* (wax moth) larvae survival model (Figure 3). When the infected larvae were treated with imipenem and mutant lytic phage Ab105-2phiΔCI, the survival rate was found to be statistically significantly higher than the larvae treated with the antibiotic or the phage alone and of untreated larvae ($p < 0.05$). Similar results were obtained for meropenem but in this case. Although, larval survival was higher after the combinatory treatment than after phage only or no treatment, the difference relative to meropenem alone was not statistically significant ($p = 0.2183$). This was probably due to the higher MIC of meropenem than of imipenem (32 μg/mL *versus* 16 μg/mL) for the Ab177_GEIH-2000 strain, indicating the need to administer greater amounts of mutant lytic phage Ab105-2phiΔCI.

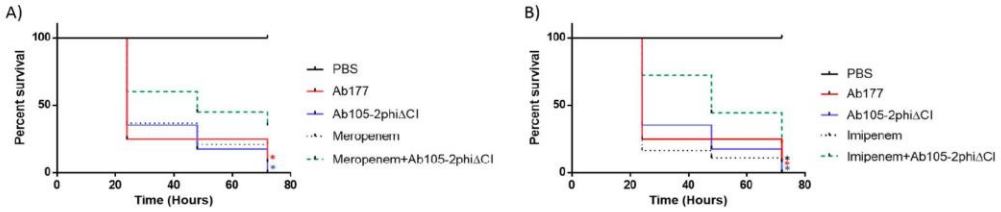

Figure 3. *G. mellonella* survival 96 h after an infection with Ab177_GEIH-2000 (1×10^5 CFU) treatment with mutant lytic phage Ab105-2phiΔCI (1×10^6 PFU) and the antibiotics meropenem at (**A**) 1/4 MIC and imipenem at (**B**) 1/4 MIC. The Log-Rank (Mantel-Cox) test, */* ($p < 0.05$) was used to compare the combination of imipenem and meropenem plus phage (line green) with each antibiotic alone (*) or the phage alone (*); *($p < 0.05$) for comparison of the combination of the phage (line green) and antibiotics (imipenem or meropenem) and untreated infection (*).

4. Discussion

Lytic phages are widely used in phage therapy, but temperate or lysogenic phages have not generally been considered suitable for the purpose, because they can enhance host competence and survival. However, temperate phages are present in almost half of bacteria that have been sequenced. Phages that are specific to pathogens causing infections can be easily identified. In addition, the problems caused by horizontal genetic transfer can now be avoided due to next generation sequencing, which enables phages to be selected, that do not pose a risk of transferring undesirable genes, such as endotoxins [15]. Temperate phages can also be easily engineered in their lysogenic state for use in phage therapy, by different means: By modifying the genes of interest as phage receptors to extend the host range; by inhibiting the lytic ability of phages without the release of endotoxins; modifying genes to enhance the killing effect of bacteriophages; increasing the life time of phages in the circulatory system of mammalians, and; transforming lysogenic phages into lytic phages [17,29–33].

In this study, we selected a temperate phage, Ab105-2phi, which did not have any toxic or virulence genes in its genome (Figure 1A1). This phage was selected with the objective of converting it into a lytic phage with potential use in phage therapy. The technique was previously described in *Salmonella enterica* bacteriophage SPN9CC and in the mycobacteriophage BPs33ΔHTH_HRM10,

recently used in a phage cocktail to treat a patient with a disseminated drug-resistant *Mycobacterium abscessus* [17,34]. The technique is based on the deletion of the CI repressor gene, which encodes the CI protein and binds to two operators, thereby repressing the *Cro* gene required for lytic development. Deleting the CI gene thus maintains the phage in a lysogenic state [10,17].

Conversion of the lysogenic into a lytic phage was confirmed, first by PCR and sequencing. Then, by the presence of clear plaques and by the infection curves for both phages: Lysogenic Ab105-phi2 and the lytic mutant Ab105-phi2ΔCI. The differences in optical density in both cases confirmed the production of a lytic mutant, and the emergence of phage resistant mutants for both phages. At MOI 10, the inhibition of growth was greater and occurred earlier than at other MOI, but resistant bacteria emerged earlier than at lower MOI, as also observed by other authors [35]. In addition, this effect was observed in the bacterial killing assays, where the growth at 6 h was greater at MOI10 than at MOI1. The mutant lytic phage also presented a latent period of 30 min, and a moderate burst size of 32 ± 2 PFU per cell was obtained with the mutant lytic phage, values in the range of those obtained in several studies for different lytic phages, including phages from *A. baumannii* [5,36–39]. The burst size is inversely related to the risk of emergence of phage resistant bacteria [40], which is one of the main objectives of phage therapy research, commonly addressed by the use of phage cocktails [41].

Although the antimicrobial activity of this mutant lytic phage was established by its ability to reduce the absorbance in a bacterial culture and also to reduce the biofilm biomass, any reduction in the development of phage resistance would increase its potential use as a therapeutic phage. In this case, the strategy we combined the phage with antibiotics [13] to enhance the potential of the Ab105-2phiΔCI phage as a therapeutic phage, and observed an almost 1 log reduction in the emergence of phage resistant mutants in the presence of the antibiotics assayed. The synergistic effect (almost 2 log decrease between the combined therapy and the compounds alone) resulted from the combination of the lytic mutant phage Ab105-2phiΔCI, and meropenem or imipenem enhanced the bactericidal effect of both the antibiotic and the phage. A strong antimicrobial effect was obtained by combining the phage at a high MOI and the antibiotic at concentrations much lower than the MIC, thereby restoring the sensitivity of the strain to imipenem and meropenem. As the host strain does not possess beta-lactamases, the resistance is probably due to the action of a Resistance-Nodulation-Division (RND) efflux pump, containing proteins that can act as phage receptor proteins. Therefore, the phage blocks the efflux pump and the antibiotic sensitivity of the strain would thus be increased [42]. The activity of the efflux pump explains the differences between antibiotics, as the efflux pumps that expulse carbapenems can act on doxycycline, when present at low levels [43].

The increase in the antimicrobial activity when the carbapenem antibiotics and the mutant lytic phage were used together was also confirmed in the survival assays with *G. mellonella*, as survival was higher in larvae that received the combined treatments. However, when the combination included meropenem (MIC, 32 μg/mL), survival was not statistically significantly higher, indicating that administration of a larger number of mutant lytic phage Ab105-2phiΔCI would be necessary (in vivo).

In conclusion, this is the first in vitro and in vivo study by which a mutant lytic phage has been used in combination with carbapenem antibiotics (imipenem and meropenem). This reduces the emergence of resistance to the phages and restores the sensitivity to antibiotics, thereby increasing the therapeutic potential of the phage. The conversion of temperate phages (with a known genomic profile) into lytic phages may provide a new source of phages for use in phage therapy.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2076-2607/7/11/556/s1. **Video 1.** Initial adsorption of phages to bacteria at 37 °C in agar slices placed directly between stainless steel O-rings for live-cell imaging. Use of extracellular DNA markers enabled lysis of more than 300 bacteria to be followed in real time.

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Conflicts of Interest: The authors declare there is no conflict of interest.

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Chapter II. *In vitro* and *in vivo* efficacy of the combination of colistin and endolysins against clinical strains of Multi-Drug Resistant (MDR) pathogens

The phage therapy can be done by the use of bacteriophages or by the use of its derived enzymes. Endolysins are highly evolved enzymes, produced by bacteriophages to digest the bacterial cell wall, that have great potential as antimicrobial agents (107).

In this chapter, we identified and characterize the two endolysins, ElyA1 and ElyA2. Besides, we determine the antimicrobial activity of ElyA1 in combination with colistin.

Two endolysins, ElyA1 and ElyA2, were identified from the genomic sequence of the *A. baumannii* temperate bacteriophages Ab1051Φ and Ab1052Φ, respectively. Those prophages are present in the genome of the *A. baumannii* clinical strain Ab105 isolated during the II Spanish Multicentre Study GEIH/REIPI-*A. baumannii* 2000-2010 (GenBank Umbrella Bioproject PRJNA422585).

Both endolysins were classified as lysozymes (N-acetylmuramidase) with a C-terminal domain corresponding with the Glycoside hydrolase 108 superfamily and also a Peptidoglycan Binding domain PG3 at the N-terminal end.

Once the endolysins were cloned and purified its muralytic activity was characterized, showing that in the case of ElyA1 the destabilization of the outer membrane with an external agent as EDTA, is necessary to the access of the enzyme to its target in the peptidoglycan agent. The high muralytic activity f this enzyme was established at pH 8.5 and 37ºC of temperature. In the case of ElyA2, no activity was seen, as an aggregative effect over the cells was observed (also in a low-level ElyA1), probably a result of a cell stress mechanism.

The antibacterial activity assays in presence of ElyA1 showed a broad spectrum of activity of this enzyme, with a high activity over all the 25 clinical strains of *A. baumannii* assayed, a more variable activity against all the 25 clinical strains of *P. aeruginosa* and a low activity over 13 of 17 clinical strains of *K. pneumoniae*.

As the endolysin ElyA1 was unable to overcome the outer membrane, its antimicrobial activity was assayed in combination with colistin, which is an antibiotic polypeptide that disturbs the outer membrane. The antimicrobial activity of colistin in combination with endolysin ElyA1 was first performed by microdilution checkerboard test to determine the minimum inhibitory concentrations (MICs) in those strains with the higher and the lower susceptibility to endolysin. Secondly, a time-kill curve assay was done in those strains whose MICs of colistin suffered at least a fourfold reduction in the previous microdilution assays. The addition of the ElyA1 resulted in a fourfold reduction in the colistin MICs in 4 of the 6 strains tested (both strains of *A. baumannii* and one each *P. aeruginosa* and *K. pneumoniae*). However, in the other strain of *P. aeruginosa,* a twofold reduction in the colistin MIC was observed and we did not detect any decrease in the other strain of *K. pneumoniae*. These results indicate a synergistic reaction between colistin and endolysin ElyA1 in all the sensitive strains to colistin. No activity was observed in those strains resistant to colistin due to the maintenance of the outer membrane.

The antimicrobial activity of the combination of ElyA1 and colistin was assayed *in vivo* in *Galleria mellonella* larvae and murine skin infection and lung infection models. The results *in vivo* confirmed the results obtained *in vitro*, thus the survival of *G. mellonella* was higher when they were treated with a combination of colistin and ElyA1. In the case of mice, the counts of bacteria were significantly reduced in those animals treated with the combination of ElyA1 and colistin in both models.

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In vitro and *in vivo* efficacy of combinations of colistin and different endolysins against clinical strains of multi-drug resistant pathogens

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The emergence of multidrug resistant (MDR) pathogenic bacteria is jeopardizing the value of antimicrobials, which had previously changed the course of medical science. In this study, we identified endolysins ElyA1 and ElyA2 (GH108-PG3 family), present in the genome of bacteriophages Ab1051Φ and Ab1052Φ, respectively. The muralytic activity of these endolysins against MDR clinical isolates (*Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) was tested using the turbidity reduction assay. Minimal inhibitory concentrations (MICs) of endolysin, colistin and a combination of endolysin and colistin were determined, and the antimicrobial activity of each treatment was confirmed by time kill curves. Endolysin ElyA1 displayed activity against all 25 strains of *A. baumannii* and *P. aeruginosa* tested and against 13 out of 17 strains of *K. pneumoniae*. Endolysin ElyA2 did not display any such activity. The combined antimicrobial activity of colistin and ElyA1 yielded a reduction in the colistin MIC for all strains studied, except *K. pneumoniae*. These results were confirmed *in vivo* in *G. mellonella* survival assays and in murine skin and lung infection models. In conclusion, combining colistin (1/4 MIC) with the new endolysin ElyA1 (350 µg) enhanced the bactericidal activity of colistin in both *in vitro* and *in vivo* studies. This will potentially enable reduction of the dose of colistin used in clinical practice.

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The worldwide emergence of multidrug resistant (MDR) microorganisms that are refractory to treatment with current therapeutic agents has highlighted the urgent need for new classes of antimicrobial agents[1]. The World Health Organization (WHO) has recently published a list of "priority pathogens" which includes those microorganisms that are considered a serious threat to human health and for which new anti-infective treatments are urgently needed. The list includes carbapenem-resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* clinical isolates[2].

One consequence of the emergence of the MDR bacteria is a return to the use of previously abandoned antimicrobials. This is the case with colistin (polymyxin E), a cationic peptide which disturbs the stability and increases the permeability of the outer membrane via electrostatic interactions and cationic displacement of the lipopolysaccharide. Although colistin exerts antimicrobial effects, it also has nephrotoxic effects and has gradually been abandoned and substituted by other, better-tolerated antibiotics[3,4]. Combining new antimicrobial agents with old antibiotics such as colistin is a new strategy in the development of novel treatments against MDR microorganisms.

In recent years, a novel drug discovery approach has explored endolysin enzymes (also referred to as enzybiotics), which are encoded by bacteriophages (viruses which infect bacteria) (5). Endolysins are actively produced during the lytic cycle and exert antibacterial activity by degrading peptidoglycan in the bacterial cell wall[5,6].

Endolysins are highly evolved enzymes produced by bacteriophages to digest the bacterial cell wall at the end of their replication cycle and release the phage progeny. Endolysins target the integrity of the cell wall and attack one of the major bonds in the peptidoglycan layer. They can be classified into five groups according to the cleavage site: N-acetyl-β-D-muramidase (lysozymes); N-acetyl-β-D-glucosaminidases (glycosidases); lytic transglycosylase; N-acetylmuramoyl-L-alanine amidases and L-alanoyl-D-glutamate endopeptidases[7,8].

Endolysins are good candidates as new antimicrobial agents against Gram-positive bacteria, in which the peptidoglycan layer of the cell wall is exposed to the medium. Several studies have evaluated the potential use of endolysins against Gram-positive bacteria such as *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* in animal models of human infections and diseases[9–16]. In Gram-negative bacteria, the outer membrane acts as a barrier to many endolysins, and very few endolysins with exogenous activity against Gram-negative bacteria have been described (many are biotechnologically engineered)[17–20]. Endolysins can attack Gram-negative bacteria when the outer membrane is previously permeabilized with agents such as EDTA, which destabilizes the lipopolysaccharides of the outer membrane; however, the combination of endolysin and EDTA is limited to topical treatment of localized infections[21,22]. In the search for alternative methods of killing MDR bacteria such as *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*, various researchers have considered increasing the muralytic activity of endolysins by combining them with different antibiotics to take advantage of synergistic responses[22,23].

In this study, we identified and characterized an endolysin, named ElyA1, isolated from the *A. baumannii* Ab105 (ROC0034a) bacteriophage Ab1051Φ. The endolysin displayed muralytic activity against a broad spectrum of MDR organisms. In addition, combining endolysin ElyA1 with colistin (polymyxin E) enhanced the susceptibility of the tested strains by at least four times (relative to the susceptibility to colistin alone), thus highlighting the potential of endolysin ElyA1 as a candidate antibacterial agent. This effect was confirmed by an *in vivo* test, in which the survival of the *G. mellonella* larvae increased when colistin (¼ MIC) was supplemented with endolysin ElyA1. Another endolysin from the same family, named ElyA2, was identified in the *A. baumannii* Ab105 bacteriophage Ab1052Φ, but did not display muralytic activity.

Results

Identification of endolysins ElyA1 and ElyA2. The 546 bp gene coding for endolysin ElyA1 was identified as an ORF (Open Reading Frame) encoding a protein of 181 aa (GenBank: ALJ99090.1) and molecular weight, 20.22 kDa (Fig. 1). The protein sequence was analysed with InterProScan and classified as a lysozyme (N-acetylmuramidase) with a C-terminal domain corresponding to the glycoside hydrolase superfamily 108 and also a peptidoglycan binding domain PG3 at the N-terminal end.

Protein homology analysis revealed a high level of homology (>80%) with a group of 9 endolysins from *A. baumannii* bacteriophages belonging to the same protein family as ElyA1[20].

The 543 bp gene coding for endolysin ElyA2 was identified as an ORF encoding a protein of 180 aa (GenBank: ALJ99174.1) and molecular weight 20.19 kDa (Fig. 1). The sequence analysis revealed that the ElyA2 protein is also a lysozyme (N-acetylmuramidase), with a C-terminal domain corresponding to the glycoside hydrolase superfamily 108, and also a peptidoglycan binding domain PG3 at the N-terminal end.

Like the ElyA1 protein, this enzyme displays a high degree of homology (>80%) with the same group of 9 endolysins and also 90% homology with the ElyA1 protein[20].

Characterization of endolysin muralytic activity. In the initial screening of the muralytic activity of the purified endolysin ElyA1 in the overlay plates with Gram-negative bacteria, a halo appeared around the lysis zones for both strains of *A. baumannii* tested (Fig. 2a).

The muralytic activity of this enzyme was characterized using the Gram-negative bacteria *A. baumannii* Ab105 as substrate, as this is the host strain for the phage Ab105Φ1. The enzymatic activity was measured after incubation at different temperatures and pH. The maximum activity was obtained after incubation for 10 min at pH8.5 and 37 °C (Fig. 2b,c). In addition, the muralytic activity on the Ab105 cells was assayed directly or after treatment of the cells with EDTA to permeabilize the outer membrane. However, no activity was detected when the enzyme was added directly to the cells whose outer membrane had not been permeabilized with EDTA, and in this case the cells also tended to aggregate (data not shown).

The antibacterial assays showed a broad lytic spectrum of activity against the strains of the three species tested (Fig. 3). As expected because of the origin of the *A. baumannii* endolysin, the activity was highest among the 25 *A. baumannii* strains tested. Although the activity was more variable in *P. aeruginosa*, muralytic activity against all of

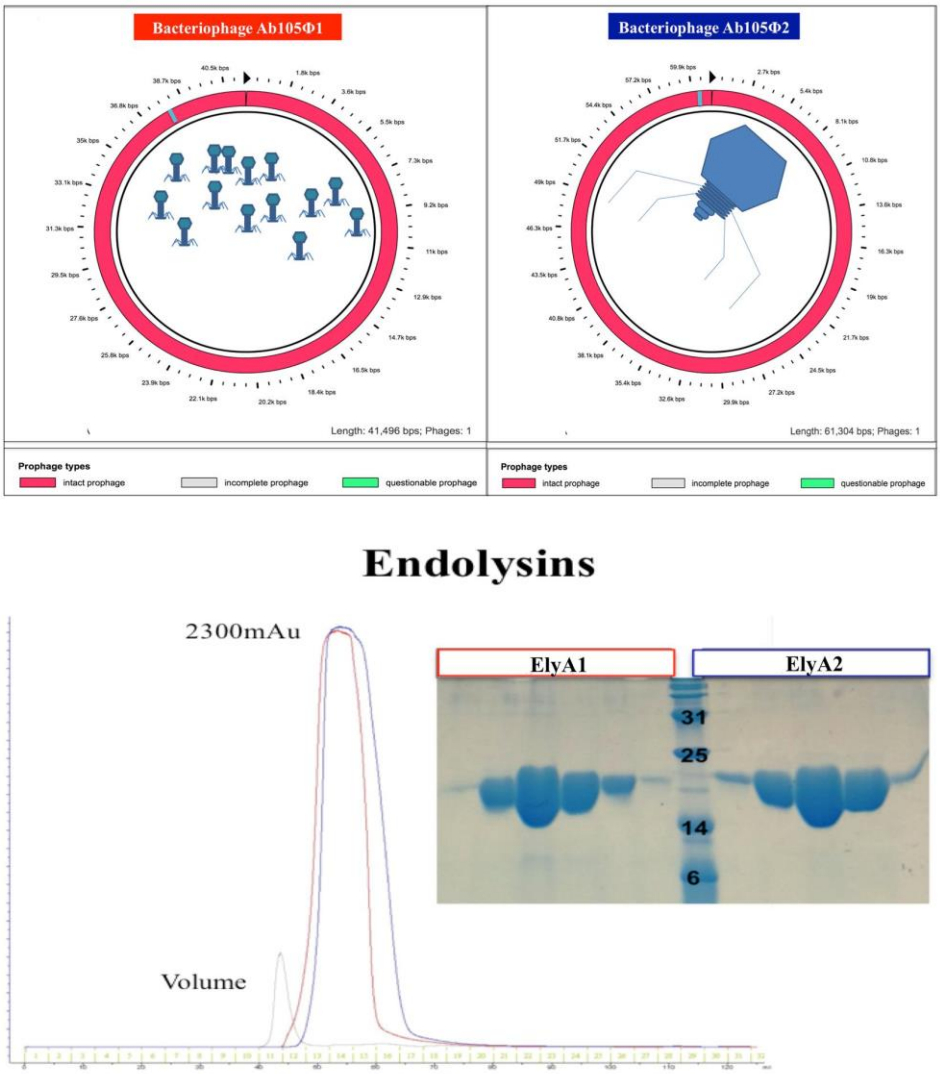

Figure 1. Genome of the bacteriophages Ab105Φ1 (GenBank: KT588074.1) and Ab105Φ2 (GenBank: KT588075.2) by figure modified with PHAST software (http://phast.wishartlab.com) (60). SDS-PAGE purification of the endolysins ElyA1 and ElyA2 (chromatographic study).

the strains was detected. Finally, endolysin ElyA1 was active against 13 of the 17 *K. pneumoniae* strains, although at lower levels than in *A. baumannii* and *P. aeruginosa*. The strains of the three species tested belonged to different strain types (STs), but the susceptibility to endolysin ElyA1 was not correlated with the ST.

Tests of the muralytic activity of endolysin ElyA2 did not detect any activity under any the conditions assayed. On the contrary, this enzyme induced aggregation of the cells at all the enzyme concentrations tested, both in the cells treated previously with EDTA and in those with an intact outer membrane.

Combined activity of endolysin ElyA1 and colistin in *in vitro* assays. As ElyA1 is only active when the outer membrane of the target bacterial cell is solubilized, the MIC of the endolysin could not be determined using the microdilution checkerboard test. We therefore aimed to detect any decrease in the colistin MICs when used in combination with endolysin ElyA1. The addition of endolysin ElyA1 yielded a fourfold reduction in the colistin MICs in four of the six strains tested (*A. baumannii* GMA001 and PON001, *P. aeruginosa* AUS531 and *K. pneumoniae* KP17) (Fig. 4). By contrast, only a twofold reduction in the colistin MIC was observed with *P. aeruginosa* AUS601 and no decrease with *K. pneumoniae* KP16. The latter was consistent with the lack of enzymatic activity observed in the antibacterial assays (Fig. 4). Finally, no antimicrobial activity was detected when the combination was tested in the colistin resistant isolates (data not shown).

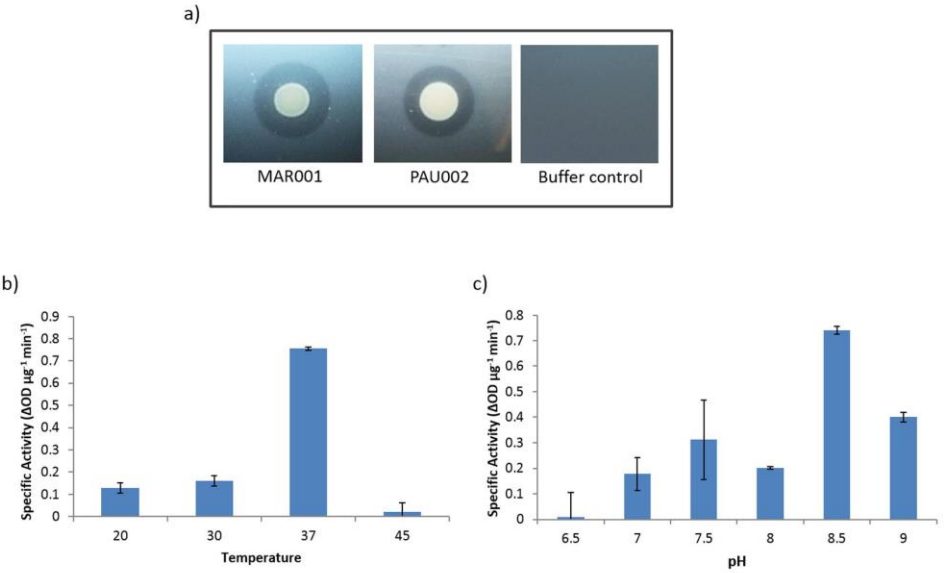

Figure 2. Characterization of enzymatic activity: (**a**) Muralytic activity of ElyA1 was determined by spotting ElyA1 and endolysin buffer as a negative control in an overlay of two Gram-negative *Acinetobacter baumannii* clinical isolates, MAR001 and PAU002; (**b**) pH range and (**c**) temperature range were determined by the specific activity, measured as the difference in optical density of the culture per µg of enzyme and minute.

The results of the time kill curve assay confirmed the results of the microdilution checkerboard test (Fig. 4). A 2 log reduction in growth of both of the *A. baumannii* strains and *P. aeruginosa* AUS531 after 6 hours in the culture with 1/4 of colistin and endolysin ElyA1 was observed, indicating a synergetic reaction between colistin and endolysin ElyA1. By contrast, there was no reduction in growth in the *K. pneumoniae* KP17 culture.

Activity of endolysin/colistin combinations in *in vivo assays*:

(a) Mortality in the *in vivo Galleria mellonella* model (Fig. 5a)
Larvae of the wax moth were infected with clinical strain *A. baumannii* GMA001. Survival of infected larvae treated with colistin (¼MIC) in combination with the ElyA1 (25 µg/ml) was significantly higher ($p < 0.05$) that that of larvae treated with colistin only (¼ MIC). Treatment with the combination of colistin (¼ MIC) and ElyA2 (25 µg/ml) did not yield significant differences ($p > 0.05$) relative to treatment with the colistin treatment, as ElyA2 did not display muralytic activity.

(b) Efficacy of ElyA 1 in the murine skin model (Fig. 5b)
Mice superficial skin wounds were infected twice (on two consecutive days) with clinical strain *A. baumannii* GMA001. The wounds were treated with colistin in combination with different doses of ElyA1 (50 µg and 350 µg), a colistin control or a buffer control. The effectiveness of the treatments was established by counting the total number of CFUs in the skin wound. The cell counts were significantly lower ($p \leq 0.05$; Student's t-test) in the colistin combination treatments (with both doses of ElyA1) than in the buffer control. The cell counts in the 350 µg ElyA1 plus colistin treatment were also significantly lower ($p \leq 0.05$; Student's t-test) than in the colistin control.

(c) Efficacy of ElyA1 in treatment of lung infection (Fig. 5c)

Infected mouse lungs were only treated with the combination of colistin and 350 µg ElyA1, as colistin plus the lower dose of endolysin (50 µg) did not display any activity in the skin infection model.

Lung CFU counts were significantly lower ($p \leq 0.05$; Student's t-test) in the mice treated with the combination of colistin and ElyA1 than in the mice treated with buffer or with colistin. There were no significant differences in the CFU counts between the buffer control group and the colistin control group.

Discussion

The discovery and development of novel antimicrobial agents to treat infections caused by the "priority" group of pathogens is a challenge facing the medical and research community[2].

Enzybiotics have become the focus of attention of many research groups worldwide. Endolysins (one type of enzybiotics) are species or genus-specific enzymes that act by hydrolysing the peptidoglycan layer of the bacterial cell wall. There are no reports of bacteria developing resistance to endolysins, which is a problem in both antibiotic therapy and phage therapy[16]. Moreover, endolysins have been recognized in the US "National Action Plan for Combating Antibiotic-resistant Bacteria"[24], which identified the use of "phage-derived lysins to kill specific bacteria while preserving the microbiota" as a key strategy to reduce the development of antimicrobial resistance due to

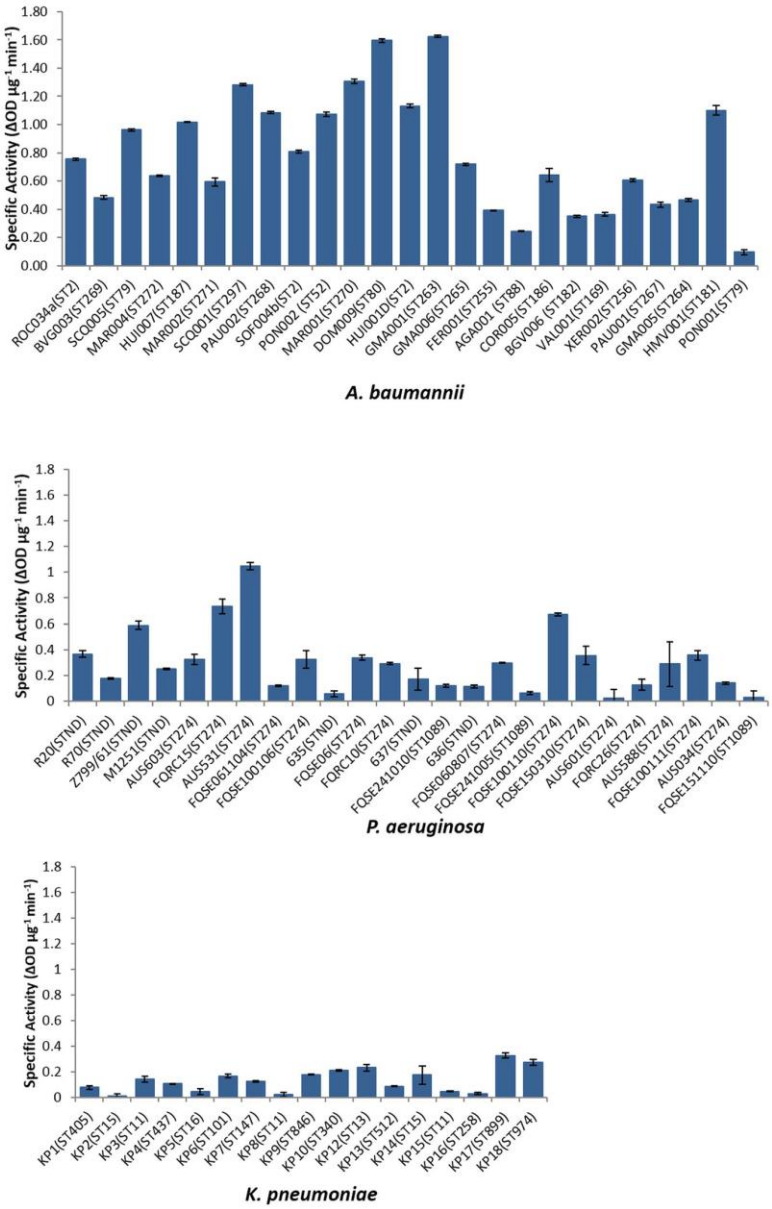

Figure 3. Specific activity of endolysin ElyA1 tested in clinical isolates from different mutlilocus sequence types (STs) of three Gram-negative members of the ESKAPE group: *Acinetobacter baumannii, Pseudomonas aeruginosa and Klebsiella pneumoniae.*

the absence of toxicity in human cells[25,26]. Moreover, some endolysins have been found to display activity against sub-populations of microbes[27,28] extracted from biofilm[29–31] and to be useful in other innovative treatments.

The outer membrane of Gram-negative bacteria acts as a barrier preventing access of many endolysins to their natural target, the peptidoglycan layer. Different strategies have been used to address this problem, including solubilization of the outer membrane with EDTA, modification of the endolysin PGs by deletion or substitution, and the development of fusion proteins such as Artilysin-175 (Art-175). This protein is made by fusing the endolysin with a peptide, successfully enabling the enzyme to pass through the outer membrane[18,32,33]. Art-175 constituted by fusing antimicrobial peptide (AMP) sheep myeloid 29-amino acid peptide (SMAP-29) with endolysin KZ144 displayed muralytic activity in a *P. aeruginosa* isolate, and continuous exposure to Art-175 did not lead to the development of resistance[18]. By itself, SMAP-29 is cytotoxic to mammalian cells[34]; however, Art-175 exhibited little toxicity in L-292 mouse connective tissue.

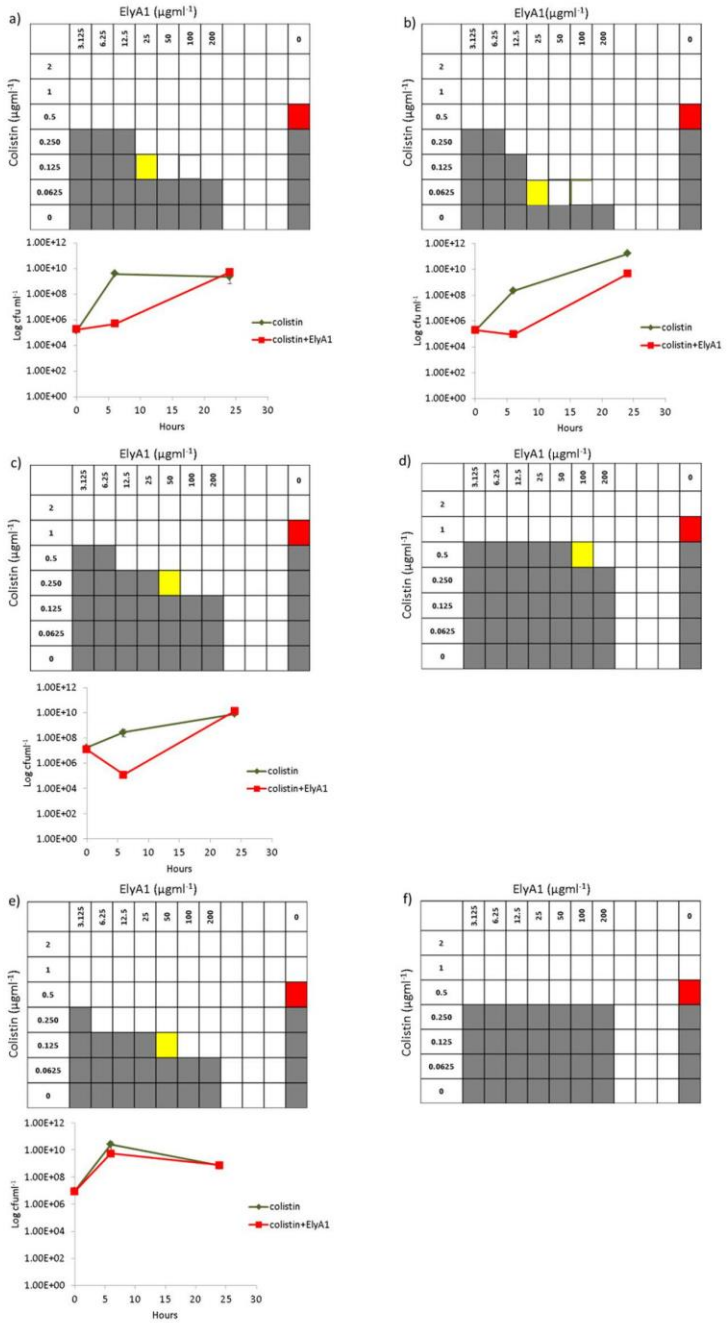

Figure 4. *In vitro* bactericidal activity of colistin in combination with endolysin ElyA1 measured by MIC and time kill curves in *Acinetobacter baumannii* strains GMA001 (**a**) and PON001 (**b**); *Pseudomonas aeruginosa* strains AUS531 (**c**) and AUS601 (**d**); *Klebsiella pneumoniae* strains KP17 (**e**) and KP16 (**f**). The time kill curves were only constructed for strains in which there was a fourfold reduction in colistin MICs (red square) when used in combination with endolysin ElyA1 (yellow square).

As a new strategy, we combined the membrane-destabilizing effect of colistin, a cationic peptide used as an active outer membrane agent (but only as a "last-line" treatment due to concerns about its nephrotoxicity and neurotoxicity[35]), and two endolysins identified by our research group and belonging to a lysozyme-like family (GH108-PG3) never before used as antimicrobial treatment.

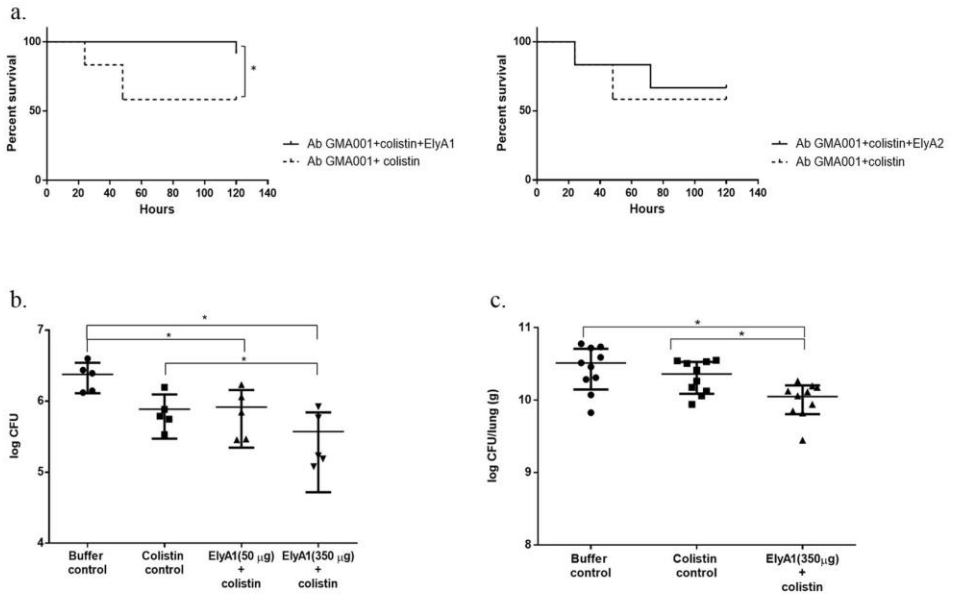

Figure 5. *In vivo* bactericidal activity of colistin in combination with endolysins ElyA1 and ElyA2. (**a**) Survival curves for *G. mellonella* larvae infected with *A. baumannii* clinical strain GMA001 and treated with colistin (1/4 MIC) and with colistin (1/4 MIC) combined with endolysin ElyA1 (25 μg/ml). Survival curves for *G. mellonella* larvae infected with *A. baumannii* clinical strain GMA001 and treated with colistin (1/4 MIC) or with colistin (1/4 MIC) combined with endolysin ElyA2 (25 μg/ml). This experiment was carried out with an appropriate survival control. *Statistically significant differences ($p < 0.05$) were determined by Graham-Breslow-Wilcoxon test (GraphPad Prism v.6); (**b**) Antimicrobial activity of endolysin ElyA1 in a murine skin model. CFU quantification in homogenized mouse skin after infection with *A. baumannii* GMA001 and treatment with colistin (1/4 MIC) in combination with different doses of endolysin ElyA1 (50 μg and 350 μg) or with buffer or colistin (controls). (**c**) Antimicrobial activity of ElyA1 in a murine lung infection model. CFU quantification in lungs after infection with *A. baumannii* GMA001 and treatment post-infection with colistin in combination with350 μg of ElyA1. * Statistically significant differences ($p < 0.05$) were determined by t-Student test (GraphPad Prism v.6).

In this study, we identified two endolysins, ElyA1 and ElyA2, obtained from *A. baumannii* bacteriophage Ab1051Φ and Ab105Φ2, available in a collection of clinical strains of *A. baumannii* isolated during the II Spanish Multicentre Study GEIH/REIPI-*A.baumannii* 2000–2010 (Accession number PRJNA422585, Genbank Umbrella Bioproject)[36,37].

Endolysins ElyA1 and ElyA2 are lysozyme-like proteins with a catalytic domain and a cell wall binding domain (CBD), responsible for recognition of the cell surface ligands and affinity for the bacterial substrate[6,38]. This structure is most commonly found in endolysins from bacteriophages that target Gram-positive bacteria. However, the PG_3 domain present in endolysins ElyA1 and ElyA2 has been identified in some Gram-negative bacteria and in a group of nine endolysins isolated from bacteriophages of *A. baumannii*; the domain shows high homology with ElyA1 and belongs to the same family (Fig. 1)[20,36,39]. The present findings regarding the molecular characteristics and comparative genomes in bacteriophage endolysins confirm previously reported findings[40].

The bacteriophages from which these endolysins were isolated, Ab1051Φ and Ab105Φ2, occur in a large number of clinical isolates of *A. baummanii*[36]. The cell wall binding domain has been shown to be responsible for the specificity and affinity of the endolysins for its substrate[39]. However, endolysin ElyA1 displayed a broader spectrum of activity against strains of *A. baumannii* and many strains of *P. aeruginosa* belonging to the same order (*Pseudomonadales*), and to lesser extent against some strains of *K. pneumoniae* from another gammaproteobacterial order, *Enterobacterales*. In this case, the target of endolysin ElyA1, identified in peptidoglycan (PG) binding domains as a D-Asn[40], is probably conserved among the *Pseudomonadales*, thus explaining the broad spectrum of action of this enzyme. Interestingly, we were not able to detect muralytic activity in endolysin ElyA2, because this enzyme induces aggregation of the cells *in vitro*. An aggregative effect was previously described in the endolysin phi12 isolated from a *S. aureus* bacteriophage, although the cause of the effect was unknown[41]. Autoaggregation has been suggested to occur in environmental stress caused by toxins, antibiotics, predation or low nutrients[42].

In the present study, we used the cationic polymyxin antibiotic colistin to overcome the impenetrability of the outer membrane to endolysin ElyA1. Colistin disturbs the outer membrane via an electrostatic interaction with lipopolysaccharides and phospholipids present in the outer membrane[4]. The synergistic effect of colistin and endolysin LysABP-01 (a lysozyme-like protein from the GH19 family) on *A. baumannii* has previously been described[22]. Although the endolysin ElyA1 does not display exogenous activity, because of its inability to cross the outer membrane, this problem was largely overcome when the enzyme was used in combination with colistin.

Strain, Plasmid, Primer, Strain	Description, Characteristics and Sequence	Origin and Reference
Strain		
Acinetobacter baumannii	25 clinical isolates (22 STs) from the II Spanish Multicentre Study (GEIH-*REIPI Acinetobacter baumannii* 2000–2010) (Accession number Genbank PRJNA422585)	27
Pseudomonas aeruginosa	25 clinical isolates (ST274 [n = 15]; ST1089 [n = 3]; ST not known [n = 7])	28
Klebsiella pneumoniae	17 clinical isolates belonging to 16 different STs	29
Escherichia coli DH5α	Strain using for cloning	Novagen
Escherichia coli Rosetta pLys-S	Strain for protein expression	Novagen
Plasmid		
pET-28a	Km^r, T7*lac*, His-Tag, T7-Tag, thrombine protease site	Novagen
Primers		
Forward	5′-AGTTCTGTTCCAGGGGCCCCATATGAACATTGAACAATATCTTGATGAA-3	This study
Reverse	5′-AGTGGTGGTGGTGGTGGTGCTCGAG**TCA**CATTGATACTCGATTAGCAAT-3′	This study

Table 1. Description of the bacterial strains, plasmids and primers used in this study. Abbreviations: ST; multilocus sequence type.

The antimicrobial activity of the combined therapy was higher than for both substances used alone, for all of the strains tested, except the *K. pneumoniae* strains. A reduction in the colistin MIC of at least fourfold was observed for all of the *A. baumannii* strains tested and for *P. aeruginosa* strain AUS531, and a corresponding twofold reduction was observed for *P. aeruginosa* strain AUS601. A reduction in the colistin MIC was also obtained for *K. pneumoniae* strain KP17, the strain most susceptible to endolysin ElyA1. The increased antimicrobial activity with endolysin ElyA1 and colistin was confirmed with an almost 3 log reduction in growth after 6 h in all strains tested, except *K. pneumoniae* KP17. Growth of the culture reached the same level as in the control after 24 h, probably due to degradation of the enzyme and colistin, as previously reported[22]. In all of the strains tested, the reduction in the colistin MIC was consistent with the muralytic activity of endolysin ElyA1 observed with those strains. No antimicrobial activity was observed when this assay was conducted with colistin-resistant strains, probably because of the inability of the enzyme to access the peptidoglycan layer, as the necessary destabilization of the outer membrane by the colistin was not produced in these isolates. However, several mechanisms of resistance to colistin have been described. In some mechanisms, the lipopolysaccharide is modified or is not produced, preventing binding of the colistin to the outer membrane. Other mechanisms include efflux pumps described in *A. baumannii* and inhibition of respiratory enzymes such as NADH oxidase in Gram-positive bacteria such as *Bacillus* spp. and NADH quinone oxidoreductase in *E. coli*. The activity of the enzyme is likely to be higher in the bacteria with colistin resistance mechanisms different from those involving modification of the lipopolysaccharides[43–49]. In Europe, the incidence of colistin resistant *A. baumannii* in intensive care units reached over 23% due to different mechanisms of resistance such as alterations in the lipopolysaccharide (LPS) as well as acquistion of *mcr* genes[50]. Because of the possible inability of these combinations to inhibit colistin resistant strains, further studies must be conducted with a range of different bacteria with different mechanisms of resistance to colistin with the aim of reducing the colistin MIC in combination with endolysin ElyA1.

The results obtained *in vitro* were confirmed with those of *in vivo* assays, as the survival of the infected *G. mellonella* larvae was higher when the worms were treated with a combination of a reduced (fourfold) MIC of colistin and endolysin ElyA1 than with colistin alone. As a control, the same assay was performed with endolysin ElyA2, in which no muralytic activity was detected, and there were no differences relative to treatment with colistin. As in *G. mellonella*, the antimicrobial activity of ElyA1 was confirmed *in vivo*. A combination of colistin and 350 µg of ElyA1 was used to treat the skin infection and lung infection in mice, yielding a significant reduction in the number of bacteria relative to treatment with colistin alone.

In conclusion, this is the first *in vitro* and *in vivo* study in which colistin has been combined with endolysin ElyA1 (glycosyde hydrolase superfamily 108) to treat infections caused by clinical MDR pathogens. This type of treatment may enable a reduction in the concentration of colistin used in antimicrobial treatments, thus also reducing the toxic side effects of the antibiotic. The broad spectrum of action of endolysin ElyA1 would enable the inclusion of more MDR Gram-negative bacteria as targets for the combined antimicrobial treatment.

Materials and Methods

Strains and culture conditions. The bacterial strains and plasmids used in this study included 25 *A. baumannii* MDR strains belonging to 22 different sequence types (STs) (Table 1). The strains were isolated from colonized or infected patients within the framework of the II Spanish Multicentre Study, in which 45 Spanish hospitals participated (GEIH-REIPI *Acinetobacter baumannii* 2000–2010, Genbank Umbrella Bioproject accession number PRJNA422585)[36,37]. The strains included 25 MDR clinical strains of *P. aeruginosa* (many included in CC274), all of which were isolated from cystic fibrosis patients, and 17 carbapenemase-producing strains of *K. pneumoniae*, which were isolated in 20 Spanish hospitals during the EuSCAPE project[51,52]. Moreover, *Escherichia coli* DH5α and Rosetta strains were used in cloning assays (Table 1).

All strains were cultured in LB (Luria-Bertani) broth at 180 rpm and 37 °C. For solid medium, 2% of agar was added to LB broth. In the transformation assays, the medium was supplemented with 50 µg/ml of ampicillin.

Identification and purification of endolysins ElyA1 and ElyA2. Endolysin gene prediction, from the genome of the bacteriophage Ab105Φ1 (GenBank: KT588074.1) and Ab105Φ2 (GenBank: KT588075.2)[36,53] (Fig. 1), was performed with the bioinformatic tools PHASTER (Phage Search Tool Enhanced Release) and RAST (Rapid Annotation Using Subsystem). Protein homology analysis was performed by BLAST (Basic Local Alignment Search Tool), Clustal Omega and MView. Protein families were assigned using InterProScan, and the domain graphic was assigned with PROSITE MyDomains.

The endolysin genes were amplified by PCR from the genomic DNA of *A. baumannii* Ab105 (which contains the DNA of the prophages Ab105Φ1 and Ab105Φ2) and cloned into the expression vector pET-28a (Novagen). The recombinant plasmids were transformed into competent *E. coli* DH5α cells (Novagen) for DNA production and purification, and the integrity of both constructs was verified by sequencing. All of the primers used are listed in Table 1. Finally, the plasmids were transformed into *Escherichia coli* Rosetta pLys-S cells (Novagen) to express the protein.

After induction with 1 mM IPTG, the culture (1 l) was grown at 30 °C for 5 h. The bacterial cells were recovered by centrifugation (in a JLA 81000 rotor, Beckman-Coulter, at 6 Krpm for 15 min) and disrupted by sonication (VibraCell 75042 sonicator, Bioblock Scientific, tip model CV33). The sample was centrifuged in a JA 25–50 rotor (Beckman-Coulter), at 20 Krpm for 30 min. The supernatant was filtered using 0.45 µm syringe-driven filters (Jet Biofil) and loaded in a His-Trap column (GE Healthcare) equilibrated with 350 mM NaCl, 50 mM Tris pH 7.5, 1 mM TCEP and 10 mM Imidazole. The proteins were eluted with 350 mM NaCl, 50 mM Tris pH 7.5, 1 mM TCEP and 150 mM Imidazole. After concentration in an Amicon Ultracel 10,000 MCWO concentrator (Millipore), the sample was loaded into a Superdex 75 16/60 column (GE Healthcare), equilibrated with 150 mM NaCl, 20 mM Tris pH 7.5 and 1 mM TCEP. The protein was eluted in a single peak. Finally, the pooled peak fractions were concentrated to 40 mg/ml, as previously described. The purification process was carried out at 4 °C, and the purity was determined by SDS-PAGE (Fig. 1).

Determination of the muralytic activity of endolysins ElyA1 and ElyA2. Muralytic activity was determined using the Gram-negative overlay method described by Schmitz *et al.*[54]. Briefly, two clinical isolates of *A. baumannii*, MAR001 and PAU002, were grown to stationary phase (10^9 CFU/ml) in LB, pelleted and resuspended in PBS buffer pH 7.4. Agar was added directly to the bacterial suspension at a concentration of 0.8%, and the mixture was autoclaved for 15 min at 120 °C. The medium containing the disorganized cells and the exposed peptidoglycan was solidified in Petri dishes, and aliquots (50 µg) of endolysin or the endolysin buffer (as a negative control) were spotted on the surface.

The muralytic activity was measured using as target a culture of *A. baumannii* Ab105 treated with EDTA in order to permeabilize the outer membrane. An overnight culture of *A. baumannii* Ab105 was diluted 1:100 in LB medium and grown to exponential phase (0.3–0.4 OD600nm). The culture was centrifuged (3000 g, 10 min), and the resulting pellet was resuspended in 20 mM Tris-HCl buffer at pH 8.5 with 0.5 mM EDTA before being incubated for 30 min at room temperature. The pellet was recovered by centrifugation and washed twice in Tris-HCl buffer pH 8.5. Finally, the cells were resuspended in 20 mM Tris-HCl 150 mM NaCl pH8.5 and 25 µg/ml of endolysin ElyA1. The activity was measured by the turbidity reduction assay, as a decrease in the optical density measured at a wavelength of 600 nm (OD_{600}) after incubation with shaking at 37 °C[17]. The OD_{600} was measured at intervals of 5 minutes for a period of 20 minutes and the time point of the highest activity was established. The optimal pH and temperature for endolysin activity were determined in the turbidity reduction assay. The reaction was carried out as previously described, with the Tris-HCl at different pH (range 6.5 to 9) and temperature (room temperature, 30 °C and 37 °C).

Antibacterial assays. The antibacterial activity of the endolysin was assayed with all of the 67 clinical strains of *A. baumannii, P. aeruginosa* and *K. pneumoniae* (Table 1). The activity was determined using the turbidity reduction assay, as previously described, at pH 8.5 and 37 °C. The incubation times in the presence of EDTA varied according to the species assayed: 30 min for *A. baumannii* and *K. pneumoniae* and 15 min for *P. pneumoniae*.

Broth microdilution checkerboard assay and microdilution test to determine minimum inhibitory concentrations (MICs). This assay was conducted with the strains disaplying the highest and the lowest susceptibility to endolysin. All the strains tested were susceptible to colistin (Table S1), except three strains, which were colistin resistant: *A. baumannii* SOF004b, *P. aeruginosa* AUS034 and *K. pneumoniae* KP2. The effect of the interaction between endolysin and colistin was determined by the microdilution checkerboard assay. Seven serial double dilutions of endolysin and 6 of colistin were made with Mueller-Hinton Broth (MHB) in the wells of a 96-well microtiter plate. The wells were then inoculated with the test culture to a final concentration of 10^5 colony forming units (cfu/ml). The MICs of colistin (0 to 2 μgml^{-1}) and of the ElyA1 protein (3.125 to 200 μgml^{-1}) were assayed independently in the same plate. The MIC was determined as the concentration of antimicrobial agent in the well in which no visible growth of bacteria was observed after incubation for 24 h at 35 °C.

Time kill curve assay. Time kill curve assays were carried out with those strains in which the colistin MIC in the colistin-ElyA1 combinations was decreased by at least fourfold in the checkerboard assays. The assay was conducted according to previously described techniques[55]. Flasks of LB containing colistin and colistin plus endolysin at the concentration indicated in the checkerboard assay were inoculated with a 1:100 dilution of an overnight culture in stationary phase of the tested strain and incubated at 37 °C and 180 rpm in a shaking incubator. Aliquots were removed after 0, 6 and 24 h and were serially diluted and plated to produce colony forming units (cfu). Synergy was established when a $\leq 2 - \log_{10}$ decrease in cells counts at 6 or 24 h in the antimicrobial combination relative to the most active single agent was observed. No effect was considered to have occurred when the counts were $<2 - \log_{10}$ lower or higher relative to the culture with the single agent. Antagonism was defined when

the counts in the culture with antimicrobial combination were $\geq 2-\log_{10}$ higher than in the culture with single most active antimicrobial agent.

The reduction in the colistin MIC in combination with endolysins was also assayed by combining colistin with another endolysin, ElyA2, isolated from bacteriophage Ab105Φ2. The curve was constructed for the same strains and under the same conditions as for colistin + ElyA1.

***Galleria mellonella* infection model.** The *Galleria mellonella* model was an adapted version of that developed by Peleg *et al.*[56] as well as in other studies with endolysins assays[57,58]. The procedure was as follows: twelve *G. mellonella* larvae, acquired from TRULARV (Biosystems Technology, Exeter, Devon, UK), were each injected with 10 µl of a suspension of *A. baumannii* GMA001, diluted in sterile phosphate buffer saline (PBS) containing 1×10^5 CFU (±0. 5 log). The injection was performed with a Hamilton syringe (volume 100 µl) (Hamilton, Shanghai, China). One hour after infection, the larvae were injected with 10 µl of colistin (1/4 MIC) plus endolysin ElyA1 (25 µg/ml), colistin (1/4 MIC) plus endolysin ELyA2 (25 µg/ml), and colistin alone (as controls), all at the same concentrations used in the time kill curve. After being injected, the larvae were placed in Petri dishes and incubated in darkness at 37 °C. The number of dead larvae was recorded during 5 days. The larvae were considered dead when they showed no movement in response to touch[56].

The mortality curves corresponding to the *in vivo Galleria mellonella* infection model were constructed using GraphPad Prism v.6, and the data were analysed using the Graham-Breslow-Wilcoxon test. In both cases, p-values < 0.05 were considered statistically significant, and the data were expressed as mean values.

Mouse skin infection model. A superficial skin wound infection by tape stripping in mouse was done as previously described[59,60], with some modifications. Female BALB/c mice (6–8 weeks old) were anaesthetized with an injection of ketamine (500 µg/mouse) and medetomidine (15 µg/mouse). Mice were shaved with an electric razor, and an area of skin of 2 cm^2 was stripped with autoclave tape, until the skin was reddish and shiny. The tape stripped areas were cleaned with ethanol and allowed to dry. The area was then treated with 10 µl of a culture of *A. baumannii* GMA001 (1×10^8 CFU/ml) or with PBS (in control mice). At 24 h post infection, the area was re-infected under the same conditions as before. The infection was established for another 24 h and the treatments were applied to groups of mice (n = 5); 3 groups were treated with colistin (1/4 MIC) in combination with 20 µl of endolysin ElyA1 (50 µg and 350 µg); a colistin control group was treated with 20 µl colistin alone (1/4 MIC); and a control group was treated with 20 µl of endolysin buffer. Three hours post-treatment the mice were euthanized with an overdose of thiopental sodium, and the skin in the wound area was excised and homogenized in sterile 0.9% NaCl, in a Retsch MM200 mixer mill. The homogenate was serially diluted and plated on agar MacConkey supplemented with ampicillin (50 µg/ml), in order to eliminate the normal skin flora, and to calculate the *A. baumannii* GMA001 CFUs in each skin sample.

Mouse lung infection model. A culture of *A. baumannii* GMA001 was grown from a 1:100 dilution of an overnight culture to an OD_{600} of 0.7. The cultures were washed and suspended in PBS to obtain an inoculum of $4–6 \times 10^7$ CFU in 40 µl per mouse.

Male BALB/c mice, 9–11 weeks old were anaesthetized by inhalation of sevofluorane (Zoetis, Madrid, Spain) and suspended by their incisors on a board in a semi-vertical position. The mice were infected by intratracheal instillation with 40 µl of a bacterial suspension ($4–6 \times 10^7$ CFUs). The mice were anaesthetized by inhalation of sevofluorane (Zoetis, Madrid, Spain) and divided into three groups (n = 10). At 3 h post-infection, the control group was treated by intranasal instillation of 30 µl endolysin buffer, the colistin control group was treated with colistin (1/4 MIC), and the treatment group was treated with a combination of colistin (1/4 MIC) and endolysin ElyA1 (350 µg). At this point, three mice were euthanized to determine the bacterial load in the lungs before treatment. Finally, 20 h after treatment, mice were euthanized with an overdose of sodium thiopental (Sandoz, Holzkirchen, Germany), and the lungs were extracted and homogenized in 1 ml of sterile 0.9% NaCl, in a Retsch MM200 mixer mill. The homogenate was serially diluted and plated on agar MacConkey for determination of the *A. baumannii* GMA001 CFUs in each lung sample.

All of the experiments with mice were conducted with the approval of and in accordance with the regulatory guidelines and standards established by the Animal Ethics Committee (INIBIC-CHUAC, Spain, project code 2016/R06).

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Author contributions

L.B., A.A., R.T., I.B., M.M., L.F.-G., developed the experiments, analysis of results and wrote manuscript. E.P.-N., F.F.-C., J.T.-C., revised the results. J.O-I., A.O., R.C., T.K., F.N., purified proteins and worked with clinical isolates. E.M., A.P., G.B., L.M.M., revised manuscript. M.T., financed and directed the experiments as well as supervised the writing of the manuscript.

Competing interests

The authors declare no competing interests.

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Chapter III. Genomic Analysis of Molecular Bacterial Mechanisms of Resistance to Phage Infection

The interest in phage therapy, as an alternative to treat MDR pathogens, has led to improve the treatment in different ways to overcome its drawbacks. One of the main disadvantages of phage therapy is the appearance of phage-resistant strains, which could provoke failure in the therapy (262). In order to have successful therapies, it is an essential objective, to previously know the resistance mechanisms of the target strain, which can be overcome by the analysis of metadata provided by WGS, which can help us to achieve *in silico* this goal.

In this study, we searched the genes associated with the phage resistance mainly focused on the CRISPR-Cas systems analysing the presence of CRISPR arrays and putative Cas proteins in 18 *A. baumannii* clinical strains from the collection of the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010" (Umbrella Bioproject PRJNA422585) belonging to the ST-2 clonal complex.

In these genomes, we detected, *in silico*, the presence of genes putatively associated with phage resistance associated to the abortive infection system, restriction-modification, newly characterized system (yet to describe the function in *A. baumannii*) and CRISPR-Cas system. We found a moderately higher presence of these genes in the strains of 2010 in comparison to those of 2000. Furthermore, we detect its presence in genomic islands (GI's) at a higher rate in the strains of 2010 compared to those of 2000.

We searched the CRISPR-Cas systems following the protocol made by Shmakov et al (263) with some modifications, due to the lack of CRISPR searching tools in metagenome data. We found 38 potential CRISPR arrays in 17 of 18 of the strains. Furthermore, we locate 705 proteins whose function could be used by CRISPR-Cas systems.

This chapter is the result of an online stay in the University of Leicester with Dr Andrew Millard learning deep analysis of sequenced metadata through a significant number of bioinformatics programs and pipelines in Linux

environments and the usage of Python language for the data processing. The chapter also constitutes a manuscript sent to *BMC Genomics*.

Research Square

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Genomic Analysis of Molecular Bacterial Mechanisms of Resistance to Phage Infection

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Abstract

Background

In order to optimize phage therapy, we need to understand how bacteria evolve against phage attack. One of the main problems of the phage therapy is the appearance of bacterial resistance variants. The use of genomics to track antimicrobial resistance is increasingly developed and used in clinical laboratories. For that reason, it is important to consider, in an emerging future with phage therapy, to detect and avoid phage resistant strains, that can be overcomed by the analysis of metadata provided by WGS. Here, we identified genes associated with phage resistance in 18 *Acinetobacter baumannii* clinical strain belonging to the ST-2 clonal complex during a decade (Ab2000 vs 2010): 9 from 2000 and 9 from 2010.

Results

The presence of genes putatively associated to phage resistance were detected. Genes detected were associated with an abortive infection system, restriction-modification system, genes predicted to be associated with defence systems but with unknown function and CRISPR-Cas system. Between 118 and 171 genes were found in the 18 clinical strains. On average, 26% of these genes were detected inside genomic islands (GIs) in the 2000 strains and 32% in 2010 strains. Furthermore, 38 potential CRISPR arrays in 17 of 18 of the strains were found, as well as 705 proteins associated with CRISPR-Cas systems.

Conclusions

A moderately higher presence of these genes in the strains of the 2010 in comparison to those of the 2000 were found, especially those related to the R-M system and CRISPR-Cas system. The presence of these genes in GIs in a higher rate in the strains of the 2010 compared to those of the 2000 was also detected. WGS and bioinformatics could be powerful tools to avoid drawbacks when a personalized therapy is applied. In this study, it allows us to take care of the phage resistance in *A. baumannii* clinical strains to prevent a failure in a possible phage therapy.

Background

As part of the ESKAPE pathogens, *A. baumannii* is frequently isolated from infections in clinical environments, and its resistance against multiple antibiotics is increasingly common (1). For this reason, it is necessary to opt for alternative treatments, such as phage therapy. However, the ability of bacteria to develop resistance mechanisms against phages is possible, even when there is no previous treatment with phage therapy due to the constant coevolutionary interactions (2). The spread of phage resistance presents a significant challenge to the efficacy of the therapy (3), (4).

It is important to know and characterize the phage resistance mechanisms of a certain species, clone or strain, prior to phage treatment in order to minimise treatment failure. Whole-genome sequencing (WGS) has been demonstrated to be a powerful tool in the detection of phage-resistance mechanisms, as well as the evolution of CRISPR-Cas arrays in bacteria subjected to phage pressure (5, 6). WGS is increasingly becoming

a cheaper and faster technology, thus it is implemented progressively in routine hospital diagnostics and research (7).

Recently, new or modified phage resistance mechanisms have been discovered and characterized (8). Although a large part of defence systems against phages are maintained over generations, there is a continuous emergence of resistance mechanisms due to spontaneous mutations as a consequence of the coexistence of phage and bacteria. Most of these mutations occur in the phage receptors proteins, employed by the phages to adhere to the cell (8). In recent years, phage resistance mechanisms are attracting increasing interest due to the rising knowledge in phage interactions with bacteria. This is leading to the discovery and characterization of new phage resistance mechanisms such as Zorya, Druantia or Thoeris (9). Phage resistance mechanisms are typically clustered in genomic “defence islands”: mobile genetic evolutionary elements that contain genes associated with phage defence systems (9, 10).

The main resistance mechanisms are related to the inhibition of the phage adsorption, blocking of phage DNA injection, cutting of the injected DNA, inhibition of the phage DNA replication, interference in the phage assembly, and bacterial suicide (11). In Fig. 1 we summarized all the characterized phage resistance mechanisms.

In this study, we focused in those which could be bioinformatically detected without any experimental process:

i) Abortive Infection systems, characterized by the fact that the phage enters the cell, but its development is interrupted in any phase (replication, transcription or translation). The mechanism of action is not entirely clear, either because of their complexity or because they are widely varied from one species to another (12).

ii) Toxin/Antitoxin systems are a specific type of ABI system but they are well-characterized and widespread through diverse species (13). In this system, a toxin is produced by the cell and is neutralized by an antitoxin. The expression of these molecules is highly controlled and varies from one system to another. When the balance between one molecule and the other is disturbed, the toxin is released and the bacteria die (14).
iii) Restriction-Modification systems consist of a restriction endonuclease and a This type of system distinguishes the DNA of the host from foreign DNA to recognize and destroy phage DNA after its injection into the When unmethylated phage DNA enters a bacteria which possesses the R-M system, it will be cleaved by the restriction endonuclease or methylated by the methyltransferase to escape the restriction (15).
iv) CRISPR-Cas (clustered regularly interspaced short palindromic repeats – CRISPR-associated system is an adaptative immune system that bacteria develop against phage DNA/RNA and other foreign DNA (16). The typical structure of the CRISPR-Cas locus is a leader sequence, followed by the repeat-spacer array and the *cas* genes operon (17). The adaptation of the CRISPR-Cas system is due to the acquisition of the spacer sequences, which are small fragments of foreign nucleic acids, between the repeats of the CRISPR locus (17). The functioning of the CRISPR-Cas system, usually divided into three steps (adaptation, processing and guidance of the crRNA-CRISPR RNA- and targeting and interference of the foreign DNA/RNA) is carried out by the Cas (CRISPR-associated) proteins (18). CRISPR-Cas systems are classified according their conserved *cas* genes and the architecture of the *cas* operon (19). Until recently, little data existed about CRISPR-Cas systems in *A. baumannii*. The pangenome analysis of *A. baumannii* has shown CRISPR-Cas

systems in the species (20). One of the most characterized systems in *A. baumannii* is the CRISPR I-Fb system (21). However, most of the Cas-related genes and CRISPR arrays are yet not identified and characterized.

In this study, we searched for putative genes associated with phage resistance and we focused on CRISPR-Cas systems by studying the CRISPR arrays and Cas protein presence through bioinformatic approach in 18 genomes of clinical strains of *A. baumannii* isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000–2010".

Methodology

Genome database

18 clinical *A. baumannii* genomes previously sequenced and annotated (II Spanish Multicenter Study, GEIH-REIPI *Acinetobacter baumannii* 2000–2010; Umbrella Bioproject PRJNA422585) (22), (23) have been studied. Nine strains were from the 2000 and nine from 2010. All of the strains belong to the ST-2 clone (22).

Search for general genes associated to bacteriophage resistance and their presence in genomic islands

In order to analyse the presence of genes putatively associated with phage resistance systems, a custom database based on genes from the "PADS Arsenal database" (https://bigd.big.ac.cn/padsarsenal/) was created (24). The genes were grouped in five systems: ABI systems related (not belonging to toxin/antitoxin system), TA systems related, R-M system related, CRISPR-Cas associated proteins and newly (NEW) characterized systems related genes. In this last category we included those genes which hit against known phage resistance genes but were associated with genes predicted to be associated with phage resistance functions and whose function in *A. baumannii* is not clear yet, such as newly characterized systems (e.g. Zorya, Druantia, Thoeris). A blast search of the complete genomes against this database and filtered out those hits which e-value > 1E-04 was made. The percentage of the genes involved in resistance was calculated by dividing the genes predicted to be associated with phage resistance by the total number of genes in the bacteria genome.

To locate Genomic Islands (GIs) three different approaches were used: IslandViewer with default settings, (25), blast search with default settings and cut-off of e-value < 1e-03 against a previously constructed ICEberg database (26) and checking the Guanine-Cytosine (GC) content of the contigs of each genome (27). The previously detected phage-resistance genes were localised in the GIs detected per genome and average percentage of genes by collection was calculated.

Search and characterization of CRISPR arrays

In a first try, CRISPRCasFinder (28) was employed but no putative CRISPR-Cas system was found. For this reason, CRISPR arrays were found using the CRISPR Recognition Tool (CRT) (29). The modification proposed by Rho et al (29) for whole-metagenomic assembled genomes called metaCRT was used (30) with

the following parameters: minimum number of repeats: 3, minimum repeat length: 12, maximum repeat length: 70, minimum spacer length: 18, maximum spacer length: 80 and with a search window of: 6.

In order to filter and validate the CRISPR arrays, a similar procedure to the first step in the protocol developed by Shmakov et al was followed (31) (Fig. 2). In the first step, CRISPR arrays separated by no more than 6 Open Reading Frames (ORF) to a putative Cas protein identified before, were considered to be part of a putative CRISPR-Cas system. Those which not were part of a putative CRISPR-Cas system were considered to be single possible CRISPR arrays. To validate these arrays, we made a short-blastn search of the spacers of the possible CRISPR arrays against all phage genomes using the INPHARED database (32). Results that were > 95% in identity and those arrays whose query hit was larger than 20 were considered putative CRISPR arrays.

Negative blast hits of the spacers of the CRISPR arrays against bacteriophage follow the procedure described by Shmakov for isolated arrays: first, arrays > 400 bp and with an ORF coverage > 0.95 were filtered out. Second, all arrays with < 850 bp that had domain in the Conserved Domain Database (CDD) search were filtered out (33), (34). Then, pairwise distances between spacers of each array were calculated (number of matches in the longest blastn hit between them, divided by the length of the smaller spacer in the pair). The spacers of each array were clustered using single linkage clustering following the same procedure as Shmakov et al. with a cut-off of 0.3. A spacer similarity index was calculated for each CRISPR array as the number of clusters formed divided by the number of spacers in the array (1 means that all the spacers are different). Those arrays whose spacer similarity index was < 0.85 were filtered out. The rest were considered putative CRISPR arrays.

To complement the procedure made by Shmakov, a search about common special low-complexity sequences that may be confused as CRISPR arrays was made, known as false-CRISPR elements (35). The presence of Tandem repeats, potentially hypermutable regions which enable bacteria to adapt to evolving environments without increasing their mutation rate, was checked with Tandem Repeat Finder (36–38). The presence of short low-complexity repeats was also examined with RepeatMasker (39). To test the results and to complete the search of low-complexity sequences a blast search against an existing false-CRISPR elements database was obtained from the CRISPRone website (35).

The search for possible Cas-related proteins was made, based on the method of Zhang et al. (40), but adding a search in “HMMCAS” website of all of their available HMM models, performed with a cut-off of reported e-values of (41). The 18 genomes were examined using hmmscan with all of the pfam HMM profiles based on NCBI entries of known Cas protein families searching in the pfam database 70 Cas-related protein families and other CRISPR-associated proteins in the pfam database (e. g. DEAD/DEAH box helicase), 93 families which were in the TIGRFAMS resource and the 24 newly characterized families (40, 42–44). For the TIGRFAMS database proteins it was necessary to build an HMM profile with hmmbuild with default settings (42) making a previous alignment for each compound of proteins with Clustal Omega version 1.2.4 (ClustalO) (45).

All of the input and output data for the searches (genes associated to phage resistance, genomic islands, CRISPR arrays and Cas proteins) were processed with Python (www.python.org) and BioPython (19304878

(46), (47).

In order to stablish the evolution and to compare the presence of the CRISPR arrays among the same clonal complex of the 18 clinical strains of *A. baumannii*, a phylogenetic tree was made using the CRISPR spacers detected. Trees were built using MEGA7 with CLUSTALW alignment (MEGA version 7.9.26) (48–50).

Results

Genes putatively associated with phage resistance in A. baumannii clinical strains and their presence in GIs

Between 118 and 171 genes were detected per genome, those could be putatively associated with bacterial defence against bacteriophages (Table 1, Additional file 1). The frequency (%) of each resistance system was calculated with the number of genes of each group divided by the total genes per genome. It was observed that the genes related to R-M and CRISPR-Cas systems showed a slightly higher prevalence in 2010 strains (Fig. 3, A). The frequency of the genes related to ABI, TA and new systems remained constant in both collections. The presence of putative phage resistance genes in GIs was also predicted (Table 2, Additional file 1), and it was found that in GIs represents in strains of the year 2010 approximately a 24% in average and approximately a 19% in the strains of the year 2000 in average (Fig. 3, B). The observed increase was produced specially in genes related to the RM system, to those related with new phage resistance mechanisms and CRISPR-Cas system.

CRISPR arrays

180 putative predicted arrays were found (without filtering) using metaCRT in the 18 genomes of ST-2 *A. baumannii* clinical strains (Table 3, Additional file 2) Post the complete process of filtering designed by Shmakov et al. (31) and removing the low-complexity sequences (35), only 40 CRISPR arrays were selected (Table 1): 18 CRISPR arrays were present in the 2000 strains and 22 in the 2010 strains. All the strains, excepting in the Ab161_GEIH-2000 strain, presented at least 1 CRISPR array.

Table 1
CRISPR arrays present in the genomes of of 18 A. baumannii clinical strains.

Strain	Contig	Size	Start	Stop	Repeat	Nº of spacers
Ab33_GEIH-2010	MSMK01000003	160	10462	10622	ATTTTGAATTTAAAA	4
Ab33_GEIH-2010	MSMK01000187	198	18280	18478	ACAAAAGAAAAAT	4
Ab49_GEIH-2010	MSMM01000317	96	1114	1210	TCATTTTGCTGTTGTT	2
Ab49_GEIH-2010	MSMM01000323	198	78	276	ACAAAAGAAAAAT	4
Ab49_GEIH-2010	MSMM01000347	122	367	489	TTTTAAATTCAAAA	3
Ab54_GEIH-2010	MSML01000240	198	3914	4112	AATTTTCTTTTCT	4
Ab54_GEIH-2010	MSML01000469	96	1108	1204	TCATTTTGCTGTTGTT	2
Ab54_GEIH-2010	MSML01000525	164	8017	8181	ATATATTTTTGA	3
Ab76_GEIH-2010	MSLY01000008	96	835	931	TCATTTTGCTGTTGTT	2
Ab76_GEIH-2010	MSLY01000677	198	3369	3567	AATTTTCTTTTCT	4
Ab76_GEIH-2010	MSLY01000708	164	714	878	ATATATTTTTGA	3
Ab103_GEIH-2010	MSLX01000655	160	9148	9308	ATTTTGAATTTAAAA	4
Ab103_GEIH-2010	MSLX01000266	164	2680	2844	ATATATTTTTGA	3
Ab103_GEIH-2010	MSLX01000388	96	1108	1204	TCATTTTGCTGTTGTT	2
Ab103_GEIH-2010	MSLX01000506	198	55	253	ACAAAAGAAAAAT	4
Ab104_GEIH-2010	MSMA01000019	96	1450	1546	TCATTTTGCTGTTGTT	2
Ab104_GEIH-2010	MSMA01000107	160	4402	4562	TTTTAAATTCAAAAT	4
Ab104_GEIH-2010	MSMA01000246	164	10815	10979	ATATATTTTTGA	3

Strain	Contig	Size	Start	Stop	Repeat	Nº of spacers
Ab105_GEIH-2010	LJHB01000001	198	125508	125706	ACAAAAGAAAAAT	4
Ab105_GEIH-2010	LJHB01000010	292	7321	7613	TAAAATAATTTTAA	5
Ab121_GEIH-2010	MSLZ01000141	198	4992	5190	AATTTTCTTTTCT	4
Ab122_GEIH-2010	MSMD01000782	164	711	875	ATATATTTTTGA	3
Ab155_GEIH-2000	LJHA01000001	198	125512	125710	ACAAAAGAAAAAT	4
Ab155_GEIH-2000	LJHA01000002	292	7323	7615	TAAAATAATTTTAA	5
Ab158_GEIH-2000	MSMC01000196	198	4027	4225	AATTTTCTTTTCT	4
Ab158_GEIH-2000	MSMC01000525	136	868	1004	ATTTTTTAATATTTA	3
Ab166_GEIH-2000	MSMG01000383	86	859	945	AAATAGCCTAAGC	2
Ab166_GEIH-2000	MSMG01001001	198	293	491	ACAAAAGAAAAAT	4
Ab166_GEIH-2000	MSMG01000974	79	1310	1389	TCTGCTGTCGGAAA	2
Ab166_GEIH-2000	MSMG01001128	194	304	498	ACGACGTGGACGATCTTC	3
Ab169_GEIH-2000	MSMF01000039	96	797	893	TCATTTTGCTGTTGTT	2
Ab169_GEIH-2000	MSMF01000336	198	152	350	ACAAAAGAAAAAT	4
Ab175_GEIH-2000	MSMI01000153	79	8115	8194	TTTCCGACAGCAGA	2
Ab175_GEIH-2000	MSMI01000682	86	2355	2441	AAATAGCCTAAGC	2
Ab177_GEIH-2000	MSME01000459	198	215	413	ACAAAAGAAAAAT	4
Ab183_GEIH-2000	MSMJ01000620	96	1077	1173	TCATTTTGCTGTTGTT	2
Ab183_GEIH-2000	MSMJ01000380	198	78	276	ACAAAAGAAAAAT	4

Strain	Contig	Size	Start	Stop	Repeat	Nº of spacers
Ab192_GEIH-2000	MSMH01000263	96	1139	1235	TCATTTTGCTGTTGTT	2
Ab192_GEIH-2000	MSMH01000273	157	0	157	TTGAATTTAAAA	4
Ab192_GEIH-2000	MSMH01000395	198	21634	21832	ACAAAAGAAAAAT	4

A phylogenetic tree of the complete CRISPR array sequences was constructed (Fig. 4), and showed an equal distribution of the spacers between the strains of the two years. Some of the spacers were predicted to be the same even in strains different year collections. Few of the arrays were unique respect to the other, such as the present in the 2000 strains Ab158_GEIH-2000_MSMC01000525, Ab166_MSMG01000383, Ab166_MSMG01000974, Ab166_MSMG01001128, Ab175_MSMI01000153 or Ab175_ MSMI01000682 However, there were 5 CRISPR arrays grouped that only were represented in the 2010 strains (Fig. 4).

Cas-related proteins

When HMM against Cas-known, Cas-related and CRISPR-associated protein families was employed, 705 Cas-related proteins were identified in the 18 genomes: 341 Cas-related proteins were detected in 2000 strains and 364 in 2010 strains (Table 4, Additional file 3). Most of them were identified as DEAD/DEAH box helicase (207 of the total) and as Type III Restriction Unit Res III (195 of 705). The vast majority of them were located next to proteins whose predicted function does not match with a Cas protein function or to proteins whose function was unknown. Other Cas-related were close in the same contig thereby giving us a clue to help identifying a functional Cas cluster. For example, in the contig MSLX01000260 from the Ab103_GEIH-2010 strain a putative Helicase_C protein (OLV37994.1) and a Cas_St_Csn2 protein (OLV37998.1) were only of 2 ORF distance between them. However, the function of the surrounding proteins was hypothetical, thus hindering the identification process as a Cas cluster.

Discussion

In clinical laboratories, genomics is rapidly being developed and utilized to track antibiotic resistance. As a result, it is critical to explore how to detect and avoid phage resistant strains, if a treatment based on phages was going to be applied, by using WGS metadata analysis. In this study, we looked for genes linked to phage resistance in 18 clinical strains of *A. baumannii*. We constructed a database with genes based in the public PADS database, as it is the most complete database about prokaryotic antiviral defence systems so far, as well as being collecting newly discovered types of defence systems to the BIG Data Center (24, 51). In this case, the high number of genes made us establish groups in order to simplify the results of the blast hits. We also tried to identify the presence of CRISPR-Cas systems by separating the search in CRISPR arrays and Cas proteins.

A difference between the presence of phage resistance genes in 2010 strains and 2000 strains was observed, with a higher presence of genes related to the RM system and CRISPR-Cas system and lower of

TA-related genes. The natural reciprocal selection pressure between host bacteria and phage increases the infectivity of the phage and the phage-resistance in the bacterium side (52). In fact, phage populations are ubiquitous at body surfaces such as lungs, intestines or skin, and they outnumber bacteria at least by 10-fold (53). In this study, the acquisition of phage resistance genes is correlated with a higher presence of complete prophages in the strains of the 2010 in comparison with those of 2000 (54). This could be a result of the development of phage resistance adaptative systems, that could promote the emergence of new phages that can overcome them, such as could happen with Ab105-1ϕ and Ab105-2ϕ, two prophages present in the 2010 collection strains but not in the 2000 collection strains (54).

Defence systems are regularly obtained by bacteria and archaea through horizontal gene transfer (HGT) owing to environmental adaptation of the bacterial communities (55, 56). We found a major average of genes acquired by HGT in the 2010 strains rather than in the 2000 ones, especially those genes related to RM system and CRISPR-Cas. It was demonstrated that only ~ 4% of RM systems are in the core genomes of prokaryotic species, suggesting they are commonly transferred (57). CRISPR-Cas systems display weak consistency within the core genome, demonstrating the prevalence of the HGT spreading this system (57, 58). The RM system and the CRISPR-Cas system commonly coexist with an elevated contribution to the bacterial immunity and they rarely operate on their own (57, 59). However, they are far from being perfect in the bacterial resistance, and phage can escape these systems by many different ways, for example the anti-CRISPR proteins (8, 60). We also observed a decreasing number of TA-related genes through the years, even their presence in GIs is higher in the 2000 strains than in the 2010's. This could be because the counteradaptation of the phage may be reached by developing antitoxin in the phage genome that inhibit the cell death and thus promote the infection of the phage (61, 62) or because they could have evolved into Cas proteins of the CRISPR-Cas system, as the TA proteins are considered as ancestors of Cas2 proteins (63).

Furthermore, we found the CRISPR-Cas genes blast hit results incomplete due to the separation in contig assembly of the genomes, which prevented us from identify proteins or arrays related to the CRISPR-Cas proteins identified in small contigs (data not shown), and also due to the high diversity of the Cas proteins and the little knowledge about these proteins in clinical strains of *A. baumannii*, which increases the difficulty identifying these type of proteins (20, 64). As a consequence, we examined the presence of CRISPR arrays and Cas proteins separately. We establish a methodology to discard false-CRISPR elements based on the method of Shmakov et al. (65) and posteriorly completed with a full evaluation of the quality of the CRISPR arrays filtered based on the search of tandem repeats, simple repeats and their presence on phage genomes (35). Secondly, another reason of developing an alternative method is the nature of the multi-resistant pathogens, their constant adaptation to different environments and thus the continuous acquisition of different mobile elements, which provokes the appearance of new CRISPR-Cas yet to be identified (66). This also fosters and extends the variability in the Cas proteins, complicating their characterization.

40 CRISPR arrays were found in the 18 *A. baumannii* clinical strains from the ST-2 clone. All of the strains presented at least one CRISPR array except one, Ab161_GEIH-2000. The vast majority of the arrays are shared between the clone ST-2 in both collections, with some exceptions such as the five arrays only found

in 2010 strains. It has been shown that the distribution of CRISPR-Cas system is MLST dependent and non-random, and thought to be a better discriminating tool than classical MLST in discriminating different *K. pneumoniae* (67, 68). On the other hand, the detection of different unique CRISPR arrays only in the 2000 strains demonstrates the dynamic interaction of these arrays throughout the years.

All of the CRISPR arrays in this study were without any Cas or putative Cas protein near to them. It was described that these “orphan” arrays belong to unknown CRISPR-Cas systems due to be to an extremely evolutionarily remote type of CRISPR-Cas (65). This existence of isolated CRISPR arrays could be explained for four reasons. First, the contig format of the studied genomes could provoke that some arrays are detected in small or incomplete contigs. Secondly, some Cas endonucleases such as Cas1 and/or Cas6 can recognize remote CRISPR arrays (69, 70). Third, it may occur the possibility of some of the unique isolated arrays form part of an undescribed CRISPR-Cas cluster extremely distant to the ones already characterized (65). And fourth, the strains may have lost the *cas* genes thus leaving the isolated arrays (65). The Cas distribution observed in this work would correspond and complete any of the hypothesis about the explanation of “orphan” CRISPR arrays mentioned before as the putative Cas proteins hit through the HMM search could form part of a complete Cas cluster. However, as it was said at the ending of the results section, it was impossible to determine *in silico* if the putative Cas detected form part of a complete and functional Cas loci.

The localization and characterization of defence systems against phages is a necessary step when designing an effective phage therapy. The WGS combined with an effective bioinformatics strategy would allow us to know what mechanisms the clinical strains have. This study shows the wide presence of genes associated with resistance against phages and their acquisition by GIs for 10 years in clinical *A. baumannii* strains from the same clonal complex ST-2 and the CRISPR arrays present on them.

Abbreviations

WGS: Whole Genome Sequencing; ST: Sequence Type; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; GI: Genomic Island; ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*; ABI: ABortive Infection; TA: Toxin Antitoxin; R-M: Restriction-Modification; GEIH*: Grupo de Estudio de Infección Hospitalaria* (Hospitalary Infection Group of Study); GC: Guanine Cytosine; CRT: CRISPR Recognition Tool; ORF: Open Reading Frames; CDD: Conserved Domain Database; HMM: Hidden Markov Model; PADS: Prokaryotic Antiviral Defence System; HGT: Horizontal Gene Transfer; MLST: Multi-Locus Sequence Typing

Declarations

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Author contributions

A.A., L.B., M.L., O.P. L.F-G., I.B., developed the analysis of results and wrote manuscript. C.O.C., A.M., revised manuscript. M.T., financed and directed the experiments as well as supervised the writing of the manuscript.

Transparency declarations

The authors have not actions to declare

Availability of data and materials

The datasets analysed during the current study are genomes of the "II Spanish Multicenter Study. GEIH-REIPI Acinetobacter baumannii 2000-2010" available in the BioProject PRJNA422585 repository with the link https://www.ncbi.nlm.nih.gov/bioproject/422585

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Figures

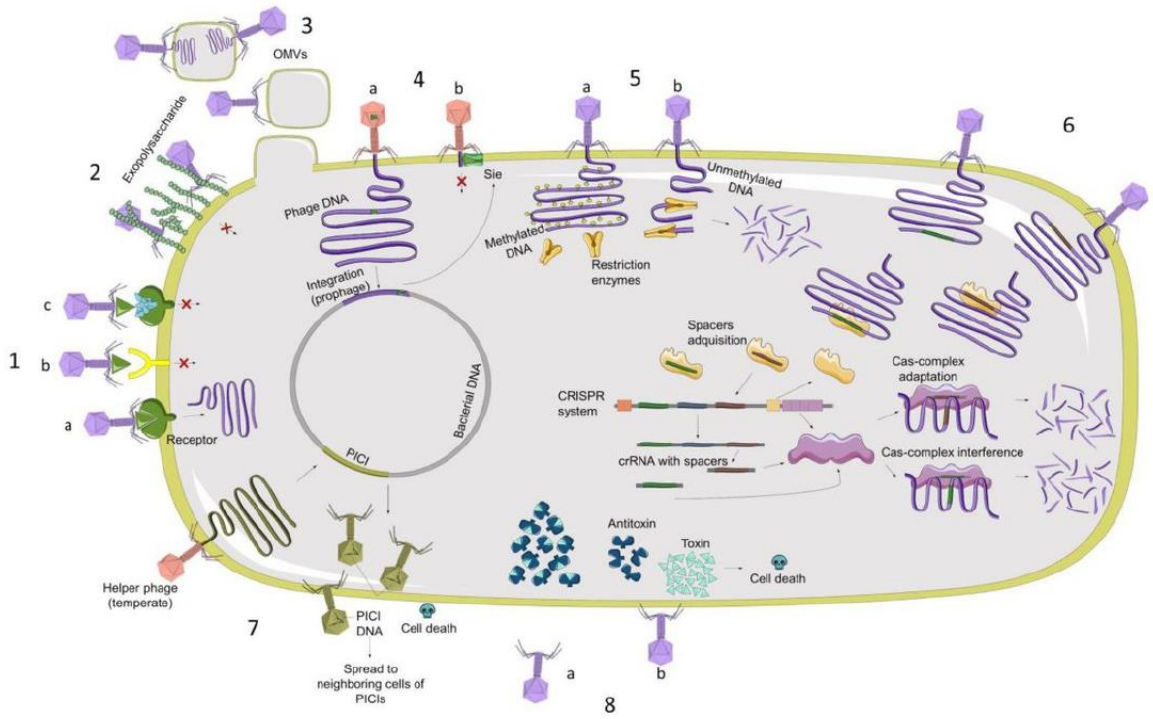

Figure 1

Representation of the main mechanisms of bacterial resistance against phage infection (from the left in a clockwise sense). 1. a: The phage recognizes the bacterial membrane receptor and can carry out the infection; b: Alterations in the receptors are produced by mutations and prevent the phage from recognizing the receptor, so it Will not infect the bacteria; c: The bacteria can block recognition by producing inhibitors that bind to receptors. 2. Production of exopolysaccharide or extracellular matrix. 3. OMVs are composed of membrane lipids, membrane proteins and periplasmic components and they are as a decoy against phages as a defence mechanism. 4. a. Bacteria block the injection of DNA from other phages, acquiring Sie systems through prophages with this type of protein; b. Once the bacterium has the prophage in its genome with the proteins that code for the Sie system, it will be able to block the entry of DNA from other phages. 5. a. The R-M system distinguishes between methylated and unmethylated DNA. Restriction enzymes cannot cut methylated DNA; b: If the phage DNA is not methylated, this system can cut the injected DNA. 6. CRISPR-Cas recognizing phage DNA sequences, incorporating them into the system and producing enzymes that are capable of recognizing these sequences to cut them. 7. The PICI system is found in the bacterial genome and induced by helper prophages to produce mature phage particles that assemble the PICI system itself to kill the infected cell and spreading this system to adjacent cells. 8. a. The most characterized Abi system is the toxin-antitoxin system. Under normal conditions, the bacterium expresses both proteins equally, so cell death does not occur; b. When the organism is subjected to stress situations, such as phage infection, the toxin is highly expressed in comparison with the antitoxin, causing cell death.

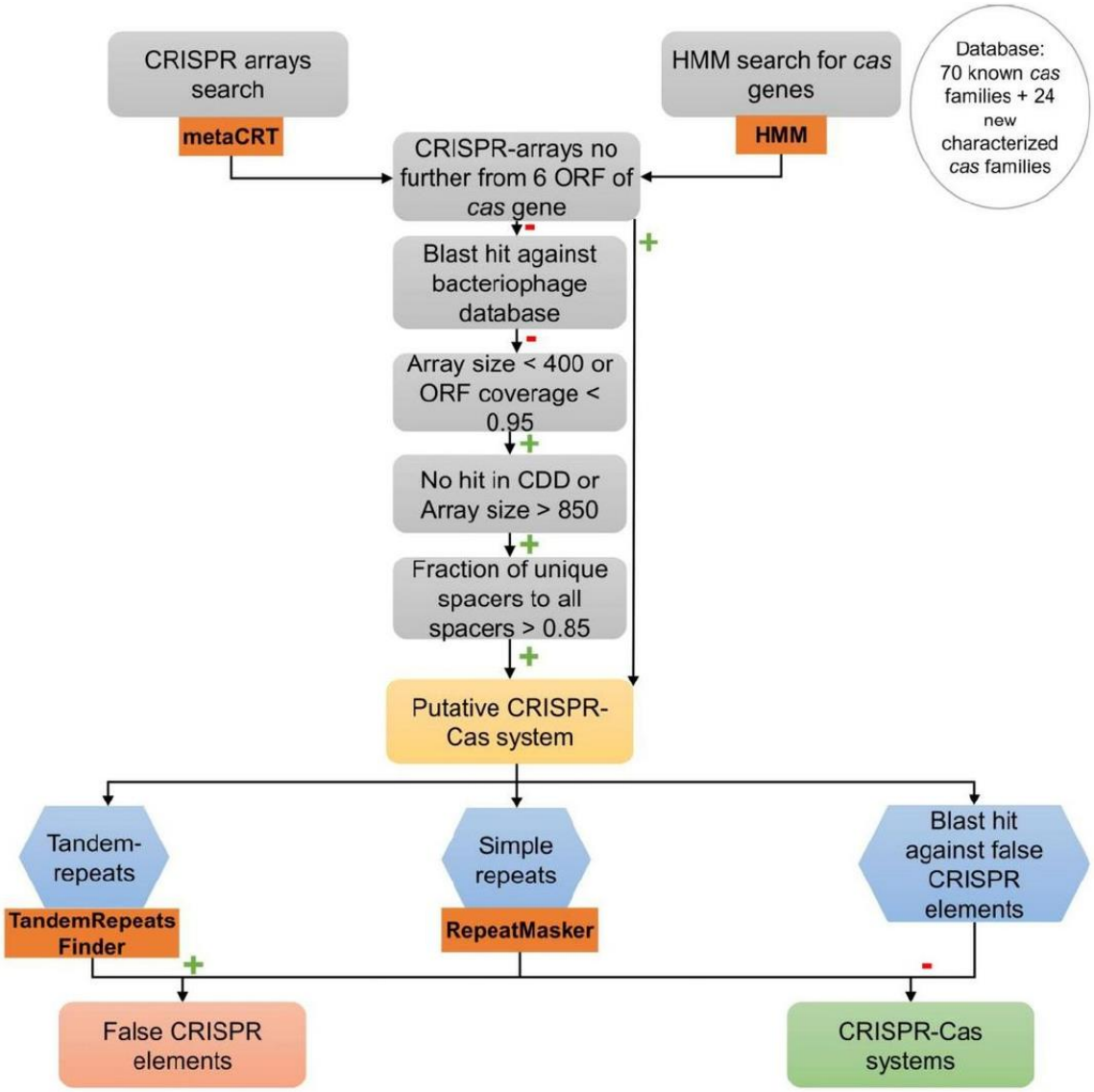

Figure 2

Search procedure of CRISPR-Cas systems in 18 genomes of A. baumannii clinical strains. Orange rectangle represents bioinformatic programmes used for that task. Green plus marks represent a positive result for each operation. Red minus marks represent a negative result for each operation.

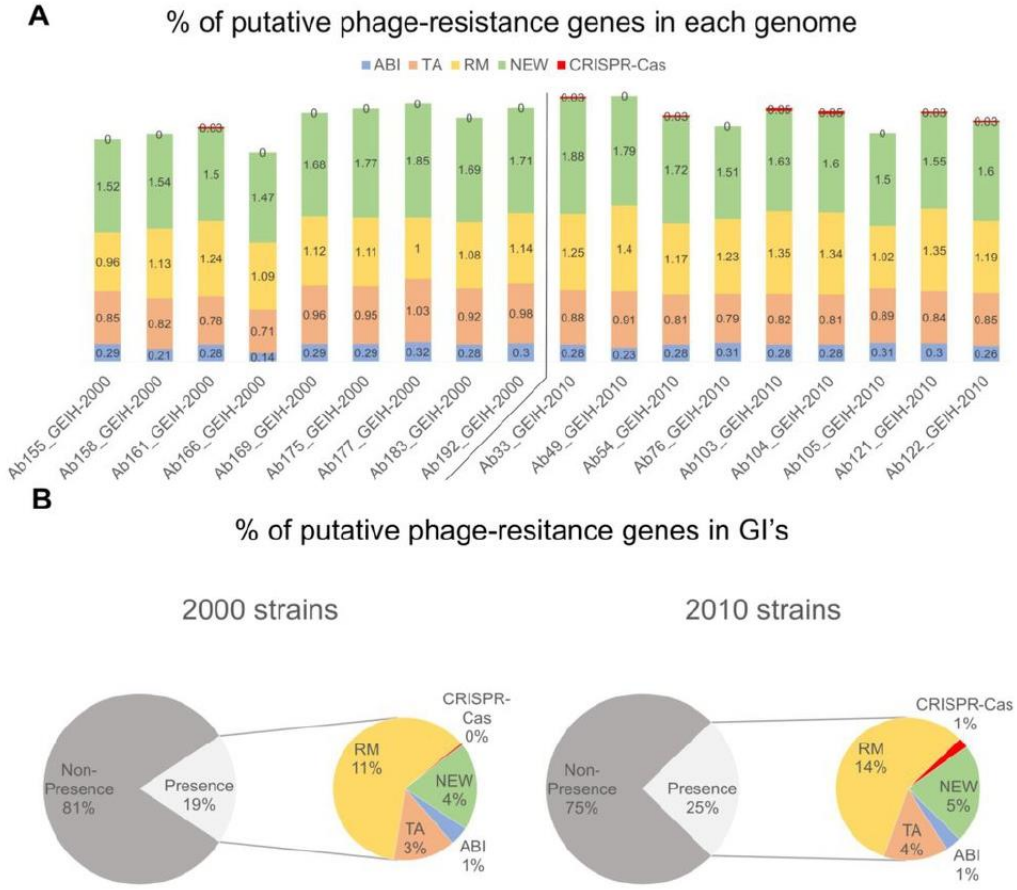

Figure 3

A. Frequency (%, rounded to two decimal numbers) of each group of genes in each genome respect to the total of genes: ABI (Abortive Infection System), TA (Toxin/Antitoxin system), RM (Restriction-Modification system) and NEW (genes associated with newly phage resistance bacterial mechanisms, e. g., Zorya, Hachiman, Druantia. B. Presence and non-presence of the putative phage resistance genes in GIs. The presence (%, rounded without decimal numbers) section is divided into the different groups of genes.

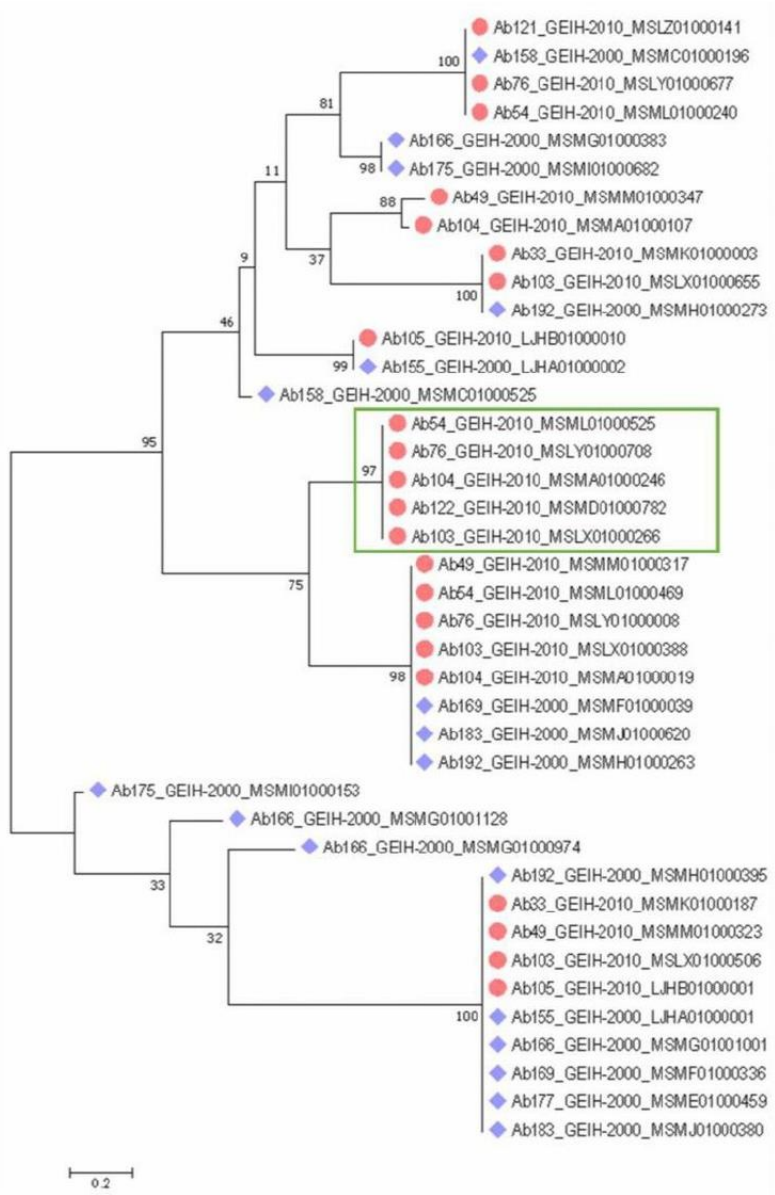

Figure 4

Phylogenetic classification of the CRISPR arrays detected in 18 genomes of A. baumannii ST-2 clinical strains through a maximum-likelihood tree with the suggested model by the MEGA analysis Tamura 3-parameter with uniform rates among sites and a bootstrap of 100. Blue rhomboid indicates that the strain belongs to the 2000 collection Red circles to the 2010 collection. Green rectangle indicates 5 CRISPR arrays only detected in the 2010 strains.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.docx
- Additionalfile2.xls
- Additionalfile3.xls

Chapter IV. Temperate bacteriophages (prophages) in *Pseudomonas aeruginosa* isolates belonging to the international Cystic Fibrosis clone (CC274)

Pseudomonas aeruginosa is a common pathogen in patients with CF. Quorum sensing (QS) systems and bacteriophages are associated with the virulence and evolution of *P. aeruginosa* during both intermittent and chronic lung infections in CF. Temperate phages have the ability to integrate into the host genome as prophages. They can confer an increase in the colonization success as they contribute to key factors like pathogenicity and biofilm formation. Furthermore, their relationship with the QS system of the bacteria may participate indirectly in this process thus favouring the phage infectivity.

In the fourth chapter, we analyse and characterize the prophages present in the genome of 24 clinical strains of *P. aeruginosa* belonging to the cystic fibrosis (CF) international clone ST274-CC274. We identified two new prophages, one inovirus and one siphovirus, the last with a gene, named *bci*, never previously described in phages that are related to the infection ability of the phage. Also, its relation with the Quorum Sensing system was analysed.

The genomic analysis of the 24 clinical isolates of *P. aeruginosa* revealed the presence of four complete prophages in three of the isolates. Three of them belonged to the filamentous phage genus of Inovirus, a group of phages that promote the formation of *P. aeruginosa* biofilm in CF lungs (264, 265). Two of the Inovirus identified, were similar to *P. aeruginosa* pf4 and pf5 Inovirus phages characteristics of *P. aeruginosa*, and the third one, present in the AUS411 isolate, was characterized as a new Inovirus-type phage. The fourth complete prophage identified, was a Siphovirus prophage present in the *P. aeruginosa* CF clinical strain AUS531.

The new Inovirus phage, identified in the isolate AUS411, was designated pf8_ST274-AUS411 (also called pf8) (GenBank accession number MN710383). The homology analysis showed a high protein homology with the pf4 Inovirus, but two proteins were characteristics of this phage, a putative toxin-antitoxin module and a methyltransferase. The Siphovirus identified in the clinical strain AUS531 was designated AUS531phi

(accession number MN585195), and the analysis of the sequence revealed the presence of the Bci (Bacteriophage Control Infection) protein, which possess a QS regulatory role. Both temperate phages, pf8 from the AUS411 strain and AUS531phi from the AUS531 strain, were observed through Transmission Electron Microscopy (TEM) confirming that the former was an Inovirus, with its characteristic filament appearance, and the second a siphovirus with a long non-contractile tail and an icosahedral capsid.

The role of the Bci protein in the interaction of the phage AUS531phi and the bacteria was analysed. For this objective a deletion of the gene was done, thus obtaining a mutant strain named AUS531Δ*bci.* This mutant strain was cultured in presence of mitomycin and the prophage, without the *bci* gene, was induced obtaining a mutant phage, AUS531phiΔ*bci*. The mutant strain was infected with the wild type phage and with the mutant phage and its effect was analysed in the expression of QS-related genes, the infection curve, motility, biofilm and pyocyanin secretion. The results demonstrated that *bci* gene increases the ability of the bacteriophage to infect *P. aeruginosa* via the regulation of QS network, decreasing the expression of the 4 genes selected (LasR, RhlR, QscR and PqsR) when the mutant strain AUS531Δ*bci* was infected with the wild type phage AUS531phi. The capacity of infection of the AUS531phi phage was compared in the infection curve, being higher when the phage carries the *bci* gene. Infection by the phage AUS531phi, containing the *bci* gene, demonstrates the relation of this phage protein with the regulation of different virulence factors in the bacteria, as a reduction in motility and an increase in biofilm production and pyocyanin secretion.

Thus, it can be concluded that the *bci* gene present in the phage AUS531phi plays a role in the infective ability of this phage regulating the host QS and virulence factors (such as pyocyanin and motility). Further studies are needed to elucidate the role of Inovirus type phage Pf8 and its putative toxin/antitoxin system and methyltransferase.

The corresponding paper at *Frontiers in Microbiology* journal is attached:

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Temperate Bacteriophages (Prophages) in *Pseudomonas aeruginosa* Isolates Belonging to the International Cystic Fibrosis Clone (CC274)

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Bacteriophages are important in bacterial ecology and evolution. *Pseudomonas aeruginosa* is the most prevalent bacterial pathogen in chronic bronchopulmonary infection in cystic fibrosis (CF). In this study, we used bioinformatics, microbiological and microscopy techniques to analyze the bacteriophages present in 24 *P. aeruginosa* isolates belonging to the international CF clone (ST274-CC274). Interestingly, we detected the presence of five members of the *Inoviridae* family of prophages (Pf1, Pf4, Pf5, Pf6, Pf7), which have previously been observed in *P. aeruginosa*. In addition, we identified a new filamentous prophage, designated Pf8, in the *P. aeruginosa* AUS411.500 isolate belonging to the international CF clone. We detected only one prophage, never previously described, from the family *Siphoviridiae* (with 66 proteins and displaying homology with PHAGE_Pseudo_phi297_NC_016762). This prophage was isolated from the *P. aeruginosa* AUS531 isolate carrying a new gene which is implicated in the phage infection ability, named Bacteriophage Control Infection (*bci*). We characterized the role of the Bci protein in bacteriophage infection and in regulating the host Quorum Sensing (QS) system, motility and biofilm and pyocyanin production in the *P. aeruginosa* isogenic mutant AUS531 Δ*bci* isolate. The findings may be relevant for the identification of targets in the development of new strategies to control *P. aeruginosa* infections, particularly in CF patients.

Keywords: prophages, inovirus, siphovirus, *Pseudomonas*, CC274 clone, cystic fibrosis

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative microorganism and a multidrug-resistant (MDR) pathogen. It is the main pathogen that causes chronic respiratory infection in cystic fibrosis (CF) and is associated with substantial morbidity and mortality in CF patients.

Bacteriophages are bacterial viruses that infect bacteria. Phages generally undergo a lytic (virulent) or lysogenic (temperate) life cycle. Lytic phages enter host cells and subsequently lyse and kill them, releasing phage progeny into the surrounding medium. Temperate phages possess the ability to go through a lysogenic cycle, entering the host cell and integrating their nucleic acid in the host genome or residing in the host cells as prophages, potentially existing in a stable state for generations until induced to start a lytic cycle (Clokie et al., 2011).

Bacteriophages in the family *Inoviridae* (inoviruses) have been described in *P. aeruginosa* biofilms and as promoters of biofilm formation (Whiteley et al., 2001; Webb et al., 2004; Knezevic et al., 2015; Secor et al., 2015). Numerous studies have shown the relationship between CF clinical isolates and Pf filamentous prophages (Finnan et al., 2004; Kirov et al., 2007; Manos et al., 2008; Mathee et al., 2008; Winstanley et al., 2009; Fothergill et al., 2012), which are long, narrow, tubular phages (about 2 μm in length and 6–7 nm in diameter) with positive-sense single-stranded circular DNA (Secor et al., 2015). Pf phages are inoviruses and usually become integrated in the chromosome of *P. aeruginosa*, although there are some exceptions, such as Pf1, which can replicate without being integrated in the host strain (Secor et al., 2015).

The pathogenic potential of *P. aeruginosa* is probably due to a combination of many different virulence factors. Several studies suggest that these factors are regulated by Quorum Sensing (QS) systems and/or bacteriophages (Lee and Zhang, 2015). The QS network in this pathogen consists of a series of connected circuits, i.e., LasI/LasR, RhlI/RhlR, QscR and PqsABCDEH/PqsR, which are regulated by molecules known as acyl-homoserine lactones (Wilder et al., 2011; Lee and Zhang, 2015; Papenfort and Bassler, 2016). Detection of these molecules indicates that *P. aeruginosa* is growing as a biofilm within the lungs of CF patients (Bjarnsholt and Givskov, 2007; Wilder et al., 2009; Winstanley and Fothergill, 2009). This bacterium permanently colonizes the lungs of CF patients, despite antibiotic treatment being administered. Microscope studies of sputum samples from these patients show that *P. aeruginosa* frequently resides within biofilms (Bjarnsholt and Givskov, 2007). Specific detection of *P. aeruginosa* via QS signaling may help to identify the agents involved in biofilm formation.

Quorum sensing systems and bacteriophages are associated with virulence and evolution of bacteria during both intermittent and chronic lung infections in CF. Some studies have shown the existence of bacteriophages in the sputum of CF patients (Ojeniyi et al., 1991; Fothergill et al., 2011), supporting the hypothesis that the bacteriophages play a role in respiratory infections in these patients. A strain of *P. aeruginosa* known as the Liverpool epidemic strain (LES) shows greater resistance to antibiotics than other strains isolated from CF patients. Genomic analysis of isolate LESB58 has demonstrated the presence of several prophages that increase the success of colonization by this *P. aeruginosa* strain as they form part of the accessory genome, the genes of which contribute to pathogenicity (Winstanley et al., 2009).

Relationships between QS and bacteriophage infection have been analyzed by several authors. Phage φpa3 has been proved to transduce mutations in QS genes in P. aeruginosa PAO1 (Monson et al., 2011). Moreover, it was demonstrated that QS systems may protect bacteria from bacteriophage infection reducing the phage receptor numbers at the stationary phase in *Escherichia coli* (Tan et al., 2015). In *Vibrio cholerae*, QS was demonstrated to control the change from a lysogenic cycle to a lytic one in the vibrio phage VP882 by QS-related genes encoded by the bacteriophage itself 30554875 (Silpe and Bassler, 2019).

In the present study, 24 sequences of *P. aeruginosa* isolates belonging to the international CF clone (ST274-CC274) were analyzed. A new filamentous prophage, designated Pf8, was identified in isolate AUS411, and analysis of its genome revealed a toxin/antitoxin system. Moreover, a new prophage from the *Siphoviridae* family was identified in isolate AUS531, which harbors a new gene that favors phage infectivity and bacterial QS control, that was named Bacteriophage Control Infection (*bci*).

MATERIALS AND METHODS

CF Clinical Isolates

All isolates (9 from CF Australian patients and 15 from Spanish patients from different clinical units), previously classified as belonging to CC274, were respiratory tract isolates from CF patients, except PAMB148, which was a blood sample. Isolates were recovered during an 18-year period (1995–2012) and included sequential isolates from several patients (López-Causapé et al., 2017). The antibiotic susceptibility profile and main antibiotic resistance-related mutations were previously analyzed (López-Causapé et al., 2017).

Genome Sequencing and Analysis of the Isolates Belonging to the ST274 Clonal Complex (CC274)

Next Generation Sequencing (NGS) was performed in a previous study, with the MiSeq sequencing system (Illumina platform) (López-Causapé et al., 2017). The sequences were assembled using the Newbler Roche assembler and Velvet (Velvet v1.2.10[1]). Putative Open Reading Frames (ORFs) were predicted using the GeneMarkS gene prediction program (Lukashin and Borodovsky, 1998). The Blast2Go and RAST servers (Conesa et al., 2005; Aziz et al., 2008) were used for functional annotation of each predicted protein. Reconstructed phage sequences were analyzed using PHAST and PHASTER tools (Zhou et al., 2011; Arndt et al., 2016). All phage proteins detected were manually annotated using the Protein BLAST (Kent, 2002), HHpred tools (Söding et al., 2005), and InterProScan tools (Zdobnov and Apweiler,

[1]https://www.ebi.ac.uk/~{}zerbino/velvet/

2001) and were found to display ≥50% protein homology. Genome sequences of the AUS531phi phage and Pf8_ST274-AUS411 filamentous phage were constructed with the assistance of CSAR-web (Chen and Lu, 2018) and RAST (Aziz et al., 2008).

The presence of the *bci* gene in prophages throughout the NCBI Nucleotide sequence was checked using BLAST, and its presence in a prophage was confirmed by PHASTER analysis of the bacterial genome. Protein domains of the protein were searched with CD-search in BLAST. Promoter regions were predicted with BPROM tool of SoftBerry[2].

Isolation of Clinical Temperate Phages From the ST274 Clonal Complex (CC274)

An overnight culture of the clinical *P. aeruginosa* isolate AUS531 was diluted in Luria-Bertani (LB) medium and grown for 2.5 h until reaching an OD_{600} (optical density measured at a wavelength of 600 nm) of 0.6, before being treated with mitomycin C (MMC). MMC was added at a concentration of 10 μg/ml and the culture was incubated at 37°C and shaken at 180 rpm until the cells were lysed. The lysate was incubated in the presence of chloroform for 20 min and centrifuged at 3400 × *g* for 10 min. Finally, the supernatant was filtered through a 0.45 nm filter (Millipore).

Transmission Electron Microscopy (TEM) Examination of Temperate Phages: Inoviruses and Siphoviruses

Concentrated phage preparations were required for transmission electron microscopy (TEM). Phage particles were precipitated overnight at 4°C with polyethylene glycol (PEG6000) 3∼5% (w/v) and 0.5 M NaCl. The solution was centrifuged at 11000 × *g* at 4°C for 15 min. The pellet was resuspended with SM buffer (100 mM NaCl; 8 mM $MgSO_4$ $7H_2O$; 50 mM Tris–HCl pH 7.5) and stored at 4°C. Samples were negatively stained with 1% aqueous uranyl acetate before examination by electron microscopy (Hargreaves et al., 2013).

Characterization of Siphovirus Temperate Phage in Relation to Quorum Sensing

bci Deleted Strain and Phage Isolation

To obtain a strain without the *bci* gene for experiments and to subsequently obtain the AUS531phiΔ*bci* mutant phage, the *bci* gene was amplified with 1 kb upstream and downstream regions for deletion in the *P. aeruginosa* AUS531 isolate. The fragment was cloned into the pEX18Gm vector (GenBank: AF047518.1) (Hoang et al., 1998) using the UP_bci(*Kpn*I)/UP_Bci(*Xho*I) combination of primers for the upstream region and the DOWN_Bci(*Xho*I)/DOWN_Bci(*Bam*HI) combination for the downstream region (**Table 1**). Fragments were digested with *Kpn*I and *Xho*I restriction enzymes (upstream fragment) and *Xho*I and *Bam*HI (downstream region). Products were ligated into the pEX18Gm plasmid, and the recombinant plasmid was transformed in *E. coli* TG1 by electroporation.

[2]http://www.softberry.com

The resulting plasmid was used to transform the *P. aeruginosa* AUS531 isolate by electroporation for genomic recombination and resulting gene knockout. Recombinant colonies representing the first crossover event were obtained by gentamicin-mediated selection. Gentamicin-resistant colonies were grown overnight in LB supplemented with 15% sucrose, and they were then plated on the same medium. Secondary crossover events were confirmed by PCR and by sequencing with the primers listed in **Table 1**. The AUS531phiΔ*bci* phage was obtained from the mutant AUS531Δ*bci* strain by induction with MMC, as previously described.

Expression of the *bci* Gene in Relation to QS Genes by RT-PCR

To establish the relationship between *bci* gene and QS, we measured the *bci* gene expression in the AUS531 strain incubated in the presence of QS signals. One colony of each of *P. aeruginosa* isolates AUS531 and AUS531Δ*bci* was inoculated in LB broth and incubated overnight at 37°C under stirring at 180 rpm. The overnight culture was diluted (1:100) and allowed to grow until reaching an OD_{600} of 0.3. Aliquots of 10 μL of QS-system signals 3-Oxo-C12-HSL (Stacy et al., 2012; López et al., 2017) and N-butanoyl-L-HSL (C4-HSL, which regulates through QS) and the same volume of DMSO as used in controls were added. The samples were incubated for 1 h (Karig and Weiss, 2005; Dubeau et al., 2009; Zhang et al., 2013). RNA was extracted using the High Pure RNA Isolation kit (Roche, Germany), and the extract was treated with DNAse (Roche, Germany). The extracted RNA measured was in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). The concentration of the samples was adjusted to 50 ng/μL to yield efficiencies of 90–110% (Rumbo et al., 2013). The expression studies were carried out with Lightcycler 480 RNA Master Hydrolysis Probe (Roche, Germany), under the following conditions: reverse transcription at 63°C for 3 min, denaturation at 95°C for 30 s, followed by 45 cycles of 15 s at 95°C and 45 s at 60°C and, finally, cooling at 40°C for 30 s. In all of the experiments, the final volume was 20 μL per well (18 μL of master mix and 2 μL of RNA at 50 ng/μL). Primers and the respective Universal Probe Library (UPL) probes are listed in **Table 1**. For each isolate, expression of all genes, primers and probes was normalized relative to the reference or housekeeping gene, *proC* (Savli et al., 2003). All of the samples were analyzed in triplicate. Statistically significant differences were determined by Student's *t*-test (GraphPad Prism v.6).

In order to analyze the effect of the phage with and without *bci* gene on QS, we analyzed the expression of QS genes (*lasR*, *rhlR*, *qscR*, and *pqsR*) in AUS531Δ*bci* incubated for 30 min with AUS531phi and AUS531phiΔ*bci* phages in an early step of bacterial growth. An overnight culture was diluted (1:100) in LB broth with 10 mM MgSO4 and 10 mM $CaCl_2$ and then grown until reaching an OD_{600} of 0.2–0.4. Both wild type AUS531phi and AUS531phiΔ*bci* phages were added at a multiplicity of infection (MOI) of 10. All controls were prepared by adding the same volume of phage buffer. RNA extraction and expression studies were carried out in the same way as in the previous step. All of the samples were analyzed in triplicate. Statistically significant differences were determined by Student's *t*-test.

TABLE 1 | Primers and probes used in this study.

PCR *P. aeruginosa* AUS531 mutant (AUS531Δ*bci*)			
Primer	**Sequence (5′-3′)**	**Restriction Site**	**Reference**
UP_Bci Fow	GGGGGTACCGCACCGCAACCTCCCGATCA	*Kpn*I	This study
UP_Bci Rev	GGGCTCGAGGGCGTAACTCCGTTCGAGGG	*Xho*I	This study
DOWN_Bci Fow	GGGCTCGAGCGCCTGGCCTATTGCCGGGC	*Xho*I	This study
DOWN_Bci Rev	GGGGGATCCGTCGTCGATGATTGAGCGAA	*Bam*HI	This study
INT UP Fow	ATTGTAGTCATACTCAAGAC	–	This study
INT DOWN Rev	TGCACCGCCTTATGTGAAAG	–	This study
pEX18 Fow	GGCTCGTATGTTGTGTGGAATTGTG	–	This study
pEX18 Rev	GGATGTGCTGCAAGGCGATTAAG	–	This study
RT-qPCR *P. aeruginosa* AUS531 mutant (AUS531Δ*bci*)			
Primer	**Sequence (5′-3′)**	**UPL probe[a]**	**References**
proC_149_Fw	CTGTCCAGCGAGGTCGAG	149	Tan et al., 2015
proC_149_Rev	CCTGCTCCACCAGTGCTT		
LasR_139_Fw	GATATCGGTTATCTGCAACTGCT	139	This study
LasR_139_Rev	CCGCCGAATATTTCCCATA		
RhlR_115_Fw	TGCGTTGCATGATCGAGT	115	This study
RhlR_115_Rev	CGGGTTGGACATCAGCAT		
QscR_133_Fw	GTTCCAGCGAGAGCATCG	133	This study
QscR_133_Rev	TGGTGATCCAGAGCAGGAA		
PqsR_151_Fw	TCGACACCAAGGTGTATTGC	151	This study
PqsR_151_Rev	TCGAGAAAGCGCAGGAAG		

[a] *Universal Probe Library (UPL) (Roche, Germany; https://lifescience.roche.com/en_es/brands/universal-probe-library.html).*

Effect of the *bci* Gene Interaction Carried by Bacteriophage on the QS: Infective Capacity, Biofilm Production, Bacterial Motility and Pyocyanin Secretion

To characterize the infection curve for the bacteriophages, an overnight culture of *P. aeruginosa* AUS531Δ*bci* was diluted 1:100 in LB broth supplemented with $MgSO_4$ and $CaCl_2$ (both at a concentration of 10 mM). The mixture was incubated at 37°C at 180 rpm until reaching an $OD_{600\ nm}$ of 0.1, before being infected with the phage AUS531phi and with the phage AUS531phiΔ*bci* at a MOI of 1 and 10. Measurements were made during 6 h at 1-h intervals. Statistically significant differences were determined by Student's *t*-test (GraphPad Prism v.6) by comparing the data obtained every hour.

To study the effect on bacterial motility, an overnight culture of *P. aeruginosa* AUS531Δ*bci* was diluted 1:100 in LB broth with 10 mM $MgSO_4$ and 10 mM $CaCl_2$ until reaching an OD_{600} of 0.5∼0.6. A spot of 1 μL of a mixture of AUS531Δbci culture and each phage (wild type AUS531phi and mutant AUS531phiΔbci at a MOI of 1) was placed in plates containing LB medium and 0.3% agar supplemented with 10 mM $MgSO_4$ and 10 mM $CaCl_2$ (Clemmer et al., 2011).

To study the effect on biofilm production, we used the modified version of the biofilm formation assay (O'Toole, 2011). Briefly, an overnight culture of *P. aeruginosa* AUS531Δ*bci* was adjusted to 10^7 CFU/mL in LB broth supplemented with 10 mM $MgSO_4$, 10 mM $CaCl_2$ and 2% glucose, and 100 μL was finally added to each well of a "U"-bottom 96-well microtiter plate and incubated at 37°C for 24 h. Thirty wells were infected at MOI 10 with AUS531phi wild temperate phage and the other 30 with AUS531Δ*bci* mutant strain. Planktonic cell growth was measured at OD_{600} before being removed. The cells were rinsed three times with distilled water and then fixed at 60° for 1 h. Biofilms were stained with 125 μL of 0.4% crystal violet (CV) for 15 min, washed four times with distilled water, and the CV retained was solubilized with 125 μL of 30% acetic acid and measured at OD_{595}. Biofilm production was calculated by dividing the OD_{595} of the CV-stained culture by the OD_{600} of the growth for each well. Statistical differences were determined with a Student's *t*-test. In order to confirm integration of the temperate phages, the presence of the *bci* gene was checked by PCR in 10 isolated colonies in each biofilm assay.

To analyze pyocyanin secretion, an overnight culture of *P. aeruginosa* AUS531Δ*bci* was diluted 1:100 in 10 mL of LB broth enriched with 10 mM $MgSO_4$ and 10 mM $CaCl_2$ and then grown until an OD_{600} of 0.2 was reached. The culture was then infected with phages AUS531phi and AUS531phiΔ*bci* at 10 MOI and incubated for 6 h. The pyocyanin was extracted by adding 6 mL of chloroform to the culture and incubating the solution for 2 h at 37°C under continuous stirring at 180 rpm. Two mL of 0.2 N HCl was then added to yield a pink to deep red solution. The absorbance of this solution was measured at an OD of 520 nm. The concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant (μg/mL), were

determined by multiplying the optical density at 520 nm by 17.072 (Essar et al., 1990; Clemmer et al., 2011). Statistically significant differences were determined by Student's *t*-test (GraphPad Prism v.6).

RESULTS

Analysis of QS Network and Temperate Phages in the Genome of *P. aeruginosa* CF Clone (ST274-CC274) Isolates

We performed a genomic analysis of the 24 *P. aeruginosa* isolates belonging to the ST274 clonal complex (CC274) obtained from CF patients and of the reference *P. aeruginosa* PAO1 strain genome (GenBank: AE004091.2) (**Table 2**). Complete prophage sequences were present in three strains in the *P. aeruginosa* sequences: AUS411, AUS531, and FQSE15-1110 (**Table 2**). Three of these showed high similarity to the *Pseudomonas* Pf inovirus, constituted by 9-15 proteins in isolates AUS411, AUS531, and FQSE15-1110. The inoviruses present in isolates AUS531 and FQSE15 were similar to the *Pseudomonas* Pf4 and Pf5 inoviruses, but the prophage detected in AUS411 was a new phage, designated Pf8_ST274-AUS411 (hereinafter referred to as Pf8) (Hay and Lithgow, 2019; Li et al., 2019). The genome of the Pf8 filamentous phage is of size 10 Kb and has a total of 16 proteins and one tRNA coding region (Genbank:MN710383). It has a GC content of 58.1%. Interestingly, Pf8 showed high protein identity with the filamentous bacteriophages Pf4 (*P. aeruginosa* PAO1) and Pf5 (*P. aeruginosa* PA14) (Mooij et al., 2007) (**Figure 1A**). However, new proteins involved in viral defense were identified in the Pf8 bacteriophage, including a putative toxin-antitoxin module (Genbank: QGZ15329.1 and QGZ15330.1) and methyltransferase (Genbank: QGZ15339.1). The prophage designated AUS531phi (accession number MN585195), detected in isolate AUS531 was found to be homologous with the *Pseudomonas* Phi297 bacteriophage.

The genome of the AUS531phi prophage is almost 50 Kb in size and contains a total of 66 proteins, one tRNA coding region and 63% GC content. The genome of the AUS531phi (**Figure 1B**) carries prophage assembly proteins, such as tail shaft proteins (GenBank: QGF21321.1, QGF21325.1, QGF21326.1, QGF21327.1, QGF21328.1, QGF21330.1, QGF21331.1, and QGF21373.1), coat-related proteins (GenBank: QGF21339.1, QGF21337.1, and QGF21335.1), a portal protein (GenBank: QGF21340.1), terminase proteins (GenBank: QGF21341.1 and QGF21342.1), lysis proteins (GenBank:QGF21343.1 and QGF21344.1), an integrase (GenBank: QGF21379.1) and other phage-related proteins. The genome harbors a carbon storage regulator (Genbank: QGF21359.1) (QS regulator associated with biofilm inhibition), called Bci protein (**Figure 1B**). The *bci* gene has 372 bp and the Bci protein has 123 amino acids (**Supplementary Figures S1A,B**, respectively) with a promoter region in the upstream sequence between the nucleotides 30327 and 30372 (**Figure S1C**) (GenBank: MN585195). There is a putative *rhl-las* box with a motif CT-(N13)-AG between the nucleotides 30342 and 30358 (**Figure S1C** and **Supplementary Material**). Following a CD-search in BLAST, CsrA superfamily domain is present between amino acids 1 and 51 with an *e*-value of 2.77e-27. We analyzed the distribution of the *bci* gene among the *P. aeruginosa* genomes deposited in the NCBI database (**Table 3**). We found that this gene was present in 33 different *P. aeruginosa* strains, with high homology (>95% of protein homology in most of these sequences). Furthermore, we found (using the PHASTER search tool) that the *bci* gene was only present in prophage sequences in these strains (**Table 3**). Moreover, in 6 of the isolates, the DNA region in this gene showed high homology (>95%) with repeat sequences of previously characterized systems (**Table 3**), showing that the *bci* gene present in phages as CRISPR sequences are constructed with previously infected DNA bacteriophage fragments (Cady et al., 2011).

TABLE 2 | Cystic Fibrosis clone isolates in the study (ST274-CC274) and their complete prophage presence.

Isolate	Location	Year	Prophage	KB	ORF	Homology (PHASTER)
AUS034	Australia	2008	0	–	–	
AUS037	Australia	2008	0	–	–	
AUS410×	Australia	2007	0	–	–	
AUS411	Australia	2007	1	5.5	9	PHAGE_Pseudo_Pf1_NC_001331(9)
AUS531	Australia	2008	1	48	62	PHAGE_Pseudo_phi297_NC_016762(22)
			2	4.7	10	PHAGE_Pseudo_Pf1_NC_001331(9)
AUS588×	Australia	2008	0	–	–	
AUS601	Australia	2008	0	–	–	
AUS603	Australia	2008	0	–	–	
AUS690	Australia	2008	0	–	–	
FQRC10	Spain	1995	0	–	–	
FQRC15	Spain	1997	0	–	–	
FQRC26	Spain	1995	0	–	–	
FQSE03-1212	Spain	2012	0	–	–	
FQSE06-0403	Spain	2003	0	–	–	
FQSE06-0610	Spain	2010	0	–	–	
FQSE10-0110	Spain	2010	0	–	–	
FQSE10-0111	Spain	2011	0	–	–	
FQSE10-0503	Spain	2003	0	–	–	
FQSE15-0803	Spain	2003	0	–	–	
FQSE15-0906	Spain	2006	0	–	–	
FQSE15-1110	Spain	2010	1	7.9	15	PHAGE_Pseudo_Pf1_NC_001331(9)
FQSE24-0304	Spain	2004	0	–	–	
FQSE24-1010	Spain	2010	0	–	–	
PAMB148	Spain	2010	0	–	–	

TEM Micrographs of Bacteriophages

We confirmed the presence of *Inoviridae* type phages by TEM examination of extracts of overnight supernatant cultures of *P. aeruginosa* isolate AUS411 (**Figure 2A**). Moreover, we confirmed the presence of the *Siphoviridae* type phages AUS531phi and AUS531phiΔ*bci* by TEM examination of the preparations (**Figure 2B**). The morphology of the structures

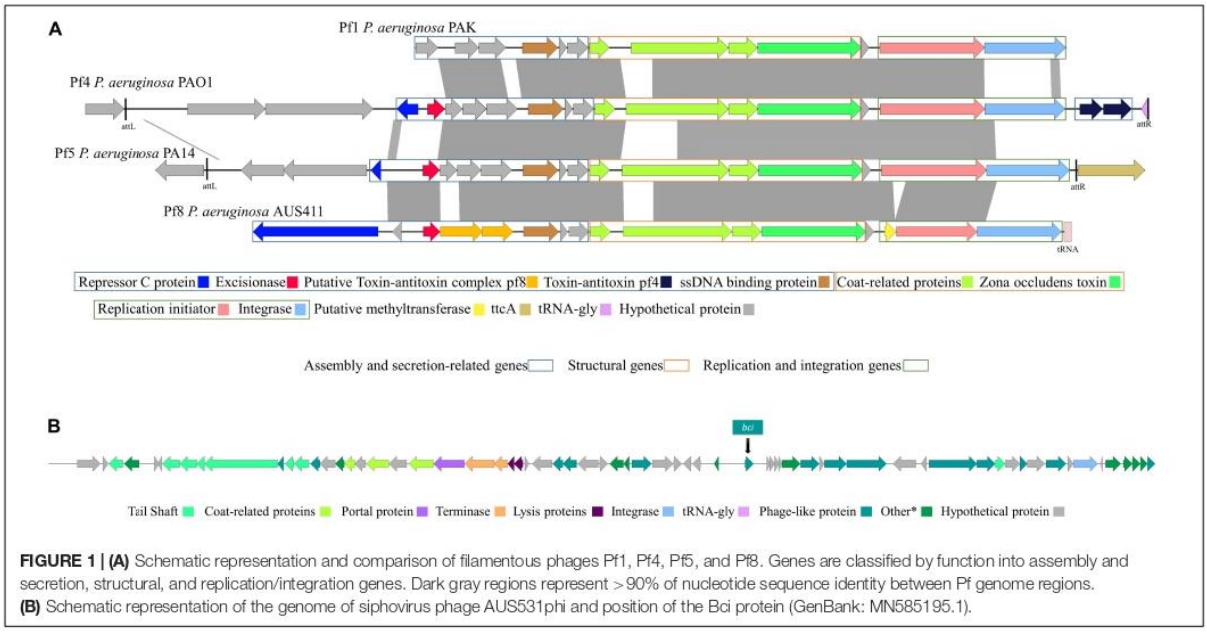

FIGURE 1 | (A) Schematic representation and comparison of filamentous phages Pf1, Pf4, Pf5, and Pf8. Genes are classified by function into assembly and secretion, structural, and replication/integration genes. Dark gray regions represent >90% of nucleotide sequence identity between Pf genome regions. **(B)** Schematic representation of the genome of siphovirus phage AUS531phi and position of the Bci protein (GenBank: MN585195.1).

seen in the pictures is clearly that of *Siphoviridae* type phages (Ališ et al., 2017).

Relationship Between the Bacteriophages and the QS System

Gene Expression

We observed an increase in the expression of the *bci* gene in the prophage region in the presence of two acyl-homoserine lactone QS inducers: 3-oxo-C12-HSL and C4-HSL (**Figure 3**), demonstrating that the *bci* gene is associated with the QS system.

In addition, we studied the effect of infection by bacteriophages AUS531phi and AUS531phiΔ*bci* in the expression of the genes *lasR*, *rhlR*, *qscR*, and *pqsR* of the QS in AUS531Δ*bci* bacterial strain to check the effect of the gene in the first step of bacteriophage infection (**Figure 4**). The graphic representation shows a fold change of around 5.0 for *lasR*, *lhlR*, and *qscR* when isolate AUS531Δ*bci* was infected with the mutant phage AUS531phiΔ*bci*, while infection with the wild phage AUS531phi yielded fold changes of around 1.0 in these genes. The differences in the expression for infection with a phage containing a *bci* gene and in the absence of this gene suggest that these genes are involved in regulating the QS system in order to overcome it and infect the bacteria. Interestingly, there was a fold change of around 13.0 in the *pqsR* gene when isolate AUS531Δ*bci* was infected with phage AUS531phiΔ*bci*, in contrast to a fold change of around 1.5 when the isolate was infected with the wild phage AUS531phi.

Infection Curve

The relationship between the *bci* gene and the ability of the phage to infect the host was demonstrated in the infection curves. The host isolate AUS531Δ*bci* grew less when infected with the wild type phage AUS531phi than when it was infected with the mutated phage, AUS531phiΔ*bci* at all the MOI assayed (**Figure 5**). The infection curves for phage AUS531phi were significantly different ($p < 0.05$) at MOI 0.1, 1 and also at MOI 10 ($p < 0.0001$). In addition, the infection curves for phage AUS531phi were significantly different from the corresponding control curves at MOI 1 and 10 ($p < 0.05$), but not at MOI 0.1. Comparison of the growth of the culture infected with the wild type phage AUS531phi and the mutant phage AUS531phiΔ*bci* revealed significant differences at MOI 0.1 and 1 ($p < 0.05$) (**Figures 5A,B**) at all time points measured, and at MOI10 the differences were significant ($p < 0.0001$) (**Figure 5C**) at 2, 3, and 4 h. These results indicate that capacity of infection of AUS531phi is higher than that of AUS531Δ*bci*, thus confirming that the *bci* gene is related to the infection capacity of this phage.

Relationship Between the Phage Infection and Virulence Factors: Motility, Biofilm and Pyocyanin Production

In order to verify the relationship between the *bci* gene from the bacteriophage and bacterial virulence, we performed motility, biofilm and pyocyanin assays. When isolate AUS531Δ*bci* was infected with the wild type phage AUS531phi, a reduction in motility was observed. By contrast, when the same isolate was infected with the *bci* gene deleted from prophage AUS531phiΔ*bci*, there was no difference in motility relative to the control (**Figure 6A**). When isolate AUS531Δ*bci* was infected with the wild type phage AUS531phi, enhanced biofilm production was observed relative to the infection of mutant phage AUS531Δ*bci* (**Figure 6B**). The PCR of the biofilm isolated colonies showed the presence of the *bci* gene in strain

TABLE 3 | Genomes of the *P. aeruginosa* isolates carrying bacteriophages with the *bci* gene and homologous CRISPR system in the *bci* gene.

Genome of *Pseudomonas* harboring bacteriophage with *bci* gene	Sequence ID	BLAST Homology (%)	Homologous Phage identified by PHASTER tool
Pseudomonas aeruginosa strain Ocean-1175	CP022525.1	99	PHAGE_Pseudo_phi297_NC_016762(25)
Pseudomonas sp. AK6U	CP025229.1	98	PHAGE_Pseudo_phi297_NC_016762(39)
Pseudomonas aeruginosa strain Pa1242	CP022002.1	98	PHAGE_Pseudo_phi297_NC_016762(21)
Pseudomonas aeruginosa strain E6130952	CP020603.1	98	PHAGE_Pseudo_phi297_NC_016762(39)
Pseudomonas aeruginosa strain N17-1	CP014948.1	97	PHAGE_Pseudo_YMC11/07/P54_PAE_BP_NC_030909(12)
Pseudomonas aeruginosa strain 97	CP031449.1	97	PHAGE_Pseudo_YMC11/02/R656_NC_028657(24)
Pseudomonas aeruginosa M18	CP002496.1	97	PHAGE_Pseudo_phi297_NC_016762
Pseudomonas aeruginosa strain AR_458	CP030327.1	97	PHAGE_Pseudo_phi297_NC_016762(23)
		95	PHAGE_Pseudo_phi297_NC_016762(15)
Pseudomonas aeruginosa strain AR439	CP029097.1	97	PHAGE_Gordon_Schwabeltier_NC_031255(62)
Pseudomonas aeruginosa strain M28A1	CP015649.1	97	PHAGE_Pseudo_YMC11/02/R656_NC_028657(20)
Pseudomonas aeruginosa strain F63912	CP008858.2	97	PHAGE_Pseudo_phi297_NC_016762(11)
Pseudomonas aeruginosa strain H5708	CP008859.2	97	PHAGE_Pseudo_YMC11/02/R656_NC_028657(26)
Pseudomonas aeruginosa RP73	CP006245.1	97	PHAGE_Pseudo_phi297_NC_016762(11)
Pseudomonas aeruginosa strain CCUG 70744	CP023255.1	96	PHAGE_Pseudo_phi297_NC_016762(29)
Pseudomonas aeruginosa strain PPF-1	CP023316.1	96	PHAGE_Gordon_Schwabeltier_NC_031255(65)
Pseudomonas aeruginosa strain F30658	CP008857.1	96	PHAGE_Pseudo_YMC11/02/R656_NC_028657(23)
Pseudomonas aeruginosa strain PB368	CP025050.1	96	PHAGE_Pseudo_phi297_NC_016762(20)
Pseudomonas aeruginosa strain PB369	CP025049.1	96	PHAGE_Pseudo_phi297_NC_016762(20)
Pseudomonas aeruginosa strain PA_150577	CP017306.1	96	PHAGE_Pseudo_YMC11/02/R656_NC_028657(27)
Pseudomonas aeruginosa strain PA121617	CP016214.1	96	PHAGE_Pseudo_YMC11/02/R656_NC_028657(20)
Pseudomonas aeruginosa strain W16407	CP008869.2	95	PHAGE_Pseudo_phi297_NC_016762(43)
Pseudomonas aeruginosa strain AR442	CP029090.1	95	PHAGE_Pseudo_phi297_NC_016762(42)
Pseudomonas aeruginosa strain T63266	CP008868.1	95	PHAGE_Pseudo_phi297_NC_016762(16)
Pseudomonas aeruginosa strain ATCC 27853	CP015117.1	96	PHAGE_Pseudo_phi297_NC_016762(37)
Pseudomonas aeruginosa DNA, complete genome, strain: 8380	AP014839.2	96	PHAGE_Pseudo_phi297_NC_016762(44)
Pseudomonas aeruginosa strain CCBH4851	CP021380.1	94	PHAGE_Pseudo_JBD44_NC_030929(31)
Pseudomonas aeruginosa strain PA7790	CP014999.1	94	PHAGE_Pseudo_phi297_NC_016762(22)
Pseudomonas aeruginosa strain PA8281	CP015002.1	94	PHAGE_Pseudo_phi297_NC_016762(22)
Pseudomonas aeruginosa strain AR_0446	CP029660.1	94	PHAGE_Pseudo_YMC11/07/P54_PAE_BP_NC_030909(25)
Pseudomonas aeruginosa PA7	CP000744.1	93	PHAGE_Pseudo_phi297_NC_016762(24)
Pseudomonas aeruginosa strain PASGNDM699	CP020704.1	92	PHAGE_Pseudo_YMC11/02/R656_NC_028657(23)
Pseudomonas aeruginosa strain PASGNDM345	CP020703.1	92	PHAGE_Pseudo_YMC11/02/R656_NC_028657(23)
Pseudomonas aeruginosa strain BAMCPA07-48	CP015377.1	89	PHAGE_Pseudo_YMC11/02/R656_NC_028657(27)
Strains with homologous CRISPR system in the *bci* gene	**Sequence ID**	**BLAST Homology (%)**	
Pseudomonas aeruginosa strain SMC4395 CRISPR repeat sequence	HQ326191.1	100	
Pseudomonas aeruginosa strain SMC4498 CRISPR repeat sequence	HQ326189.1	97	
Pseudomonas aeruginosa strain SMC4494 CRISPR repeat sequence	HQ326188.1	97	
Pseudomonas aeruginosa strain SMC4489 CRISPR repeat sequence	HQ326187.1	97	
Pseudomonas aeruginosa strain F63912	CP008858.2	97	
Pseudomonas aeruginosa RP73	CP006245.1	97	

(https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/).

AUS531Δ*bci* infected with the wild-type phage, thus confirming integration of this phage in the genome. Similarly, pyocyanin secretion was higher with the AUS531phi bacteriophage than with the AUS531phiΔ*bci* bacteriophage (**Figure 6C**) confirming that the *bci* gene influences bacterial virulence.

DISCUSSION

Cystic fibrosis is the main life-limiting recessive genetic disorder in the Caucasian population. It affects multiple organs, but is particularly damaging to the lungs. Colonization of the

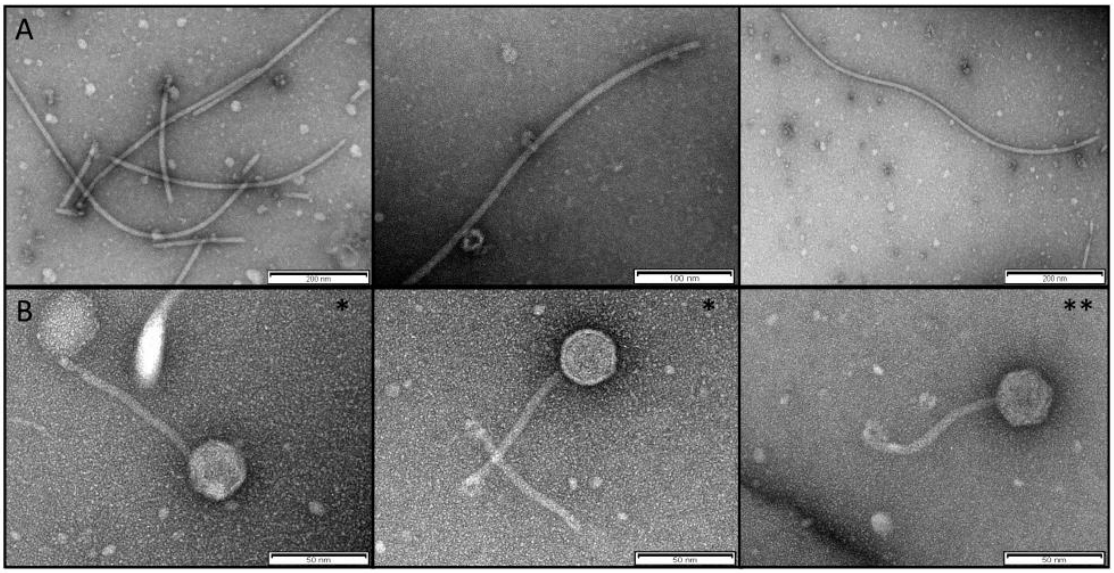

FIGURE 2 | (A) TEM of the *Inoviridae* Pf8 bacteriophage of *P. aeruginosa* clinical isolate AUS411 (Pf8_ST274-AUS411). **(B)** TEM of *Siphoviridae* bacteriophage of *P. aeruginosa* clinical isolates AUS531 (AUS531phi*) and AUS531Δ*bci* (AUS531Δ*bci*phi**).

respiratory tract by some pathogens such as *P. aeruginosa* exacerbates the severity of the disease in CF patients (Rey et al., 2018).

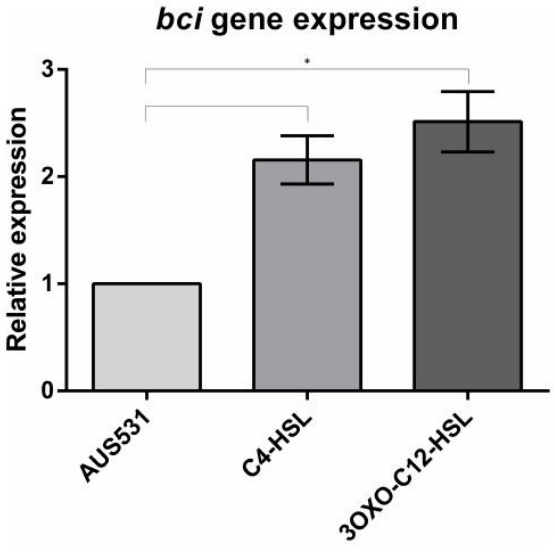

FIGURE 3 | Relative expression of the *bci* gene in AUS531 isolate under the quorum sensing inducers C4-HSL and 3OXO-C12-HSL. Standard deviations are indicated. (*) Statistically significant differences ($p < 0.05$) were determined by Student's *t*-test (GraphPad Prism v.6).

Temperate bacteriophages of *P. aeruginosa* are involved in the horizontal transfer of DNA and show selective preference for developing and accumulating in the specific conditions of the lower lung (Tariq et al., 2015). Although most phages are pathogens that kill their bacterial hosts, filamentous phages live together with their host (Mai-Prochnow et al., 2015). Filamentous phages are widely distributed in Gram-negative bacteria and they have a strong impact on the physiology, adaptation and virulence of their host bacteria, with a high presence in *P. aeruginosa* biofilms (Rice et al., 2009; Secor et al., 2015).

The high-risk clone *P. aeruginosa* ST274 is one of the most prevalent clones in CF patients (Kidd et al., 2012). Genomic

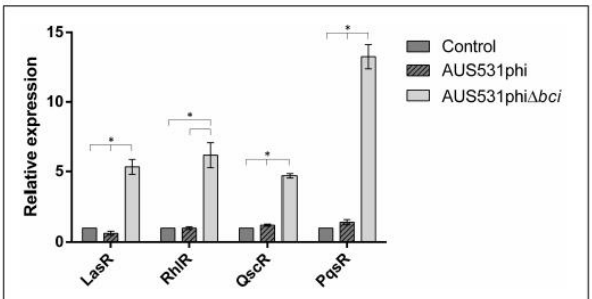

FIGURE 4 | Relative expression of quorum sensing-related genes of the AUS531Δ*bci* isolate infected with AUS531phi and AUS531phiΔ*bci* bacteriophages. Standard deviations are indicated. (*) Statistically significant differences ($p < 0.05$) were determined by Student's *t*-test (GraphPad Prism v.6).

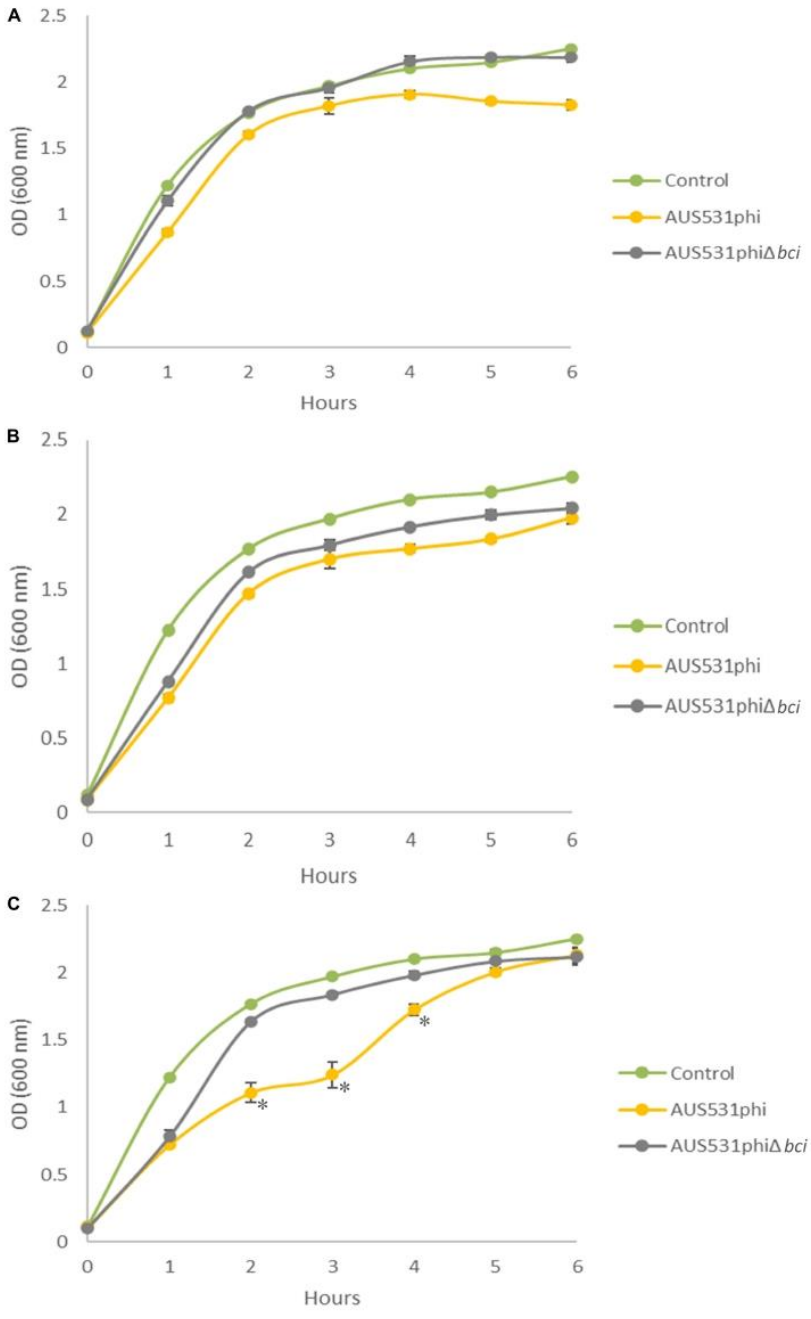

FIGURE 5 | Infection curves for the lysogenic phages AUS531phi **(A)** and AUS531phiΔ*bci* at MOIs 0.1 **(A)**, 1 **(B)**, and 10 **(C)** during 6 h. Standard deviations are indicated. Statistically significant differences were determined by Student's *t*-test for each point on the curve (GraphPad Prism v.6). (*) indicates a strongly significant difference ($p < 0.0001$).

analysis shows the presence of complete prophage regions in 3 of the 24 isolates of the *P. aeruginosa* CC274 clone. Inovirus-type phages are present in three different isolates of this clone, two corresponding to previously described phages (Knezevic et al., 2015), and one, the pf8 phage present in the AUS411 isolate, which is a new type of Pf inovirus characterized

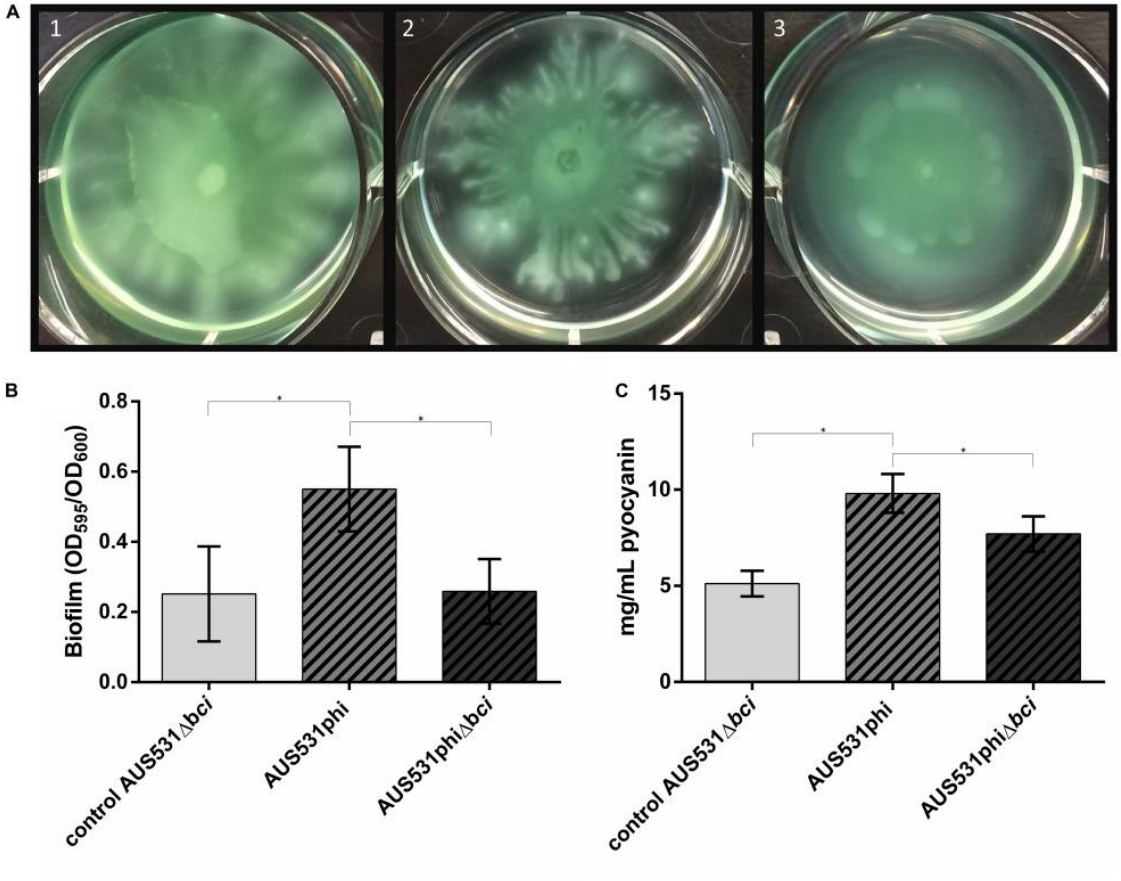

FIGURE 6 | (A) Motility assay in AUS531Δ*bci* under normal conditions (1), adding AUS531phi phage (2), and adding AUS531phiΔ*bci* phage (3). **(B)** Biofilm production after 24 h of AUS531 wild type isolate, AUS531Δ*bci* under normal conditions, AUS531Δ*bci* in response to addition of the wild type AUS531phi phage and the mutant AUS531Δ*bci* phage. (*) Statistically significant differences were determined by Student's *t*-test (GraphPad Prism v.6). **(C)** Pyocyanin production of controls AUS531 and AUS531Δ*bci* and AUS531Δ*bci* in response to infection with the wild-type strain.

by the presence of a putative toxin/antitoxin system and a methyltransferase. However, only the AUS531 isolate contains a complete siphovirus type phage, never previously described, the AUS531phi phage.

The Pf8 phage carries a putative novel type of toxin/antitoxin system (Mai-Prochnow et al., 2015), located between an excisionase (acc. no. QGZ15328.1). The genes that encode toxin-antitoxin systems are common in bacteria and are usually located adjacent to genes related to plasmids and other mobile genetic elements (DeShazer, 2004; Dziewit et al., 2007). In prophages preserve their genomes in bacterial hosts via the toxin/antitoxin system, giving them a selective advantage under different stress conditions (Wen et al., 2017).

Temperate bacteriophages can also drive host genome evolution through gene disruption, duplication, transduction or by acting as anchor points for major chromosomal rearrangements (Davies et al., 2016). Previous studies have demonstrated a possible relationship between QS signaling and regulation. The QS system is able to control anti-phage defense mechanisms, leading to lower susceptibility to phage infection in QS-proficient cells. In *Vibrio anguillarum*, QS downregulates expression of the *ompK* gene, thus increasing the resistance to phage KVP40 (Tan et al., 2015; Hoque et al., 2016). In *E. coli*, LamB phage receptors can shield isolates from attack by lytic bacteriophage λ (Høyland-Kroghsbo et al., 2013). However, bacteriophages infect bacteria with a functional QS, as in *P. aeruginosa*, because once the barrier to infection has been overcome it is advantageous for the phage to remain in the genome as a temperate phage improving cooperative behavior by eliminating QS-deficient social cheaters, which not have the phages, despite the fact that phage adsorption is higher in those with QS-deficient strain (Saucedo-Mora et al., 2017). In addition, molecular evolution of clinical strains of *Acinetobacter baumannii* has been demonstrated to have

occurred between 2000 and 2010, leading to possession of a functional quorum network and the acquisition of bacteriophages (López et al., 2018).

The QS regulatory role of the *bci* in the prophage was demonstrated by the increase in the expression of this gene in presence of two acyl-homoserine lactone QS inducers, C4-HSL and 3oxo-C12-HSL, which activate the receptors RhlR and LasR, respectively and may induce the *bci* expression (Medina et al., 2003). In the promoter region of the gene, there is a putative *rhl-las* box (Subramoni et al., 2015), that have been predicted to be upstream QS-controlled genes (Whiteley et al., 1999). Also, when an infection with the wild prophage AUS531phi and with the mutant phage AUS531phiΔ*bci* were done, the bacterial QS expression was regulated by the wild type phage, which suggest that the *bci* gene has a role in the control of the bacterial QS, favoring the infection by the temperate phages as was also observed in the infection curves.

Virulence factors as pyocyanin production, biofilm and motility are regulated by QS and also influenced by the phage infections (Morkunas et al., 2012; Hosseinidoust et al., 2013; Latino et al., 2014; Castañeda-Tamez et al., 2018; Tariq et al., 2019). The infection with the wild type phage, AUS531phi, carrying the *bci* gene, increased the production of virulence factors, pyocyanin and biofilm, whose presence is characteristic in the lung of CF patients (Castañeda-Tamez et al., 2018). The increase in both biofilm and pyocyanin and a reduction in the swarming motility, are a response to the phage infection which is higher when the *bci* gene is present, but also due to the integration of the temperate phage and the *bci* gene in the bacterial genome, as was described previously. Pyocyanin production has proven to be protective against oxidative stress environments for *P. aeruginosa* (Vinckx et al., 2010). The higher pyocyanin production may be due to a protective response to a higher infectivity capacity of the phage AUS531phi. Temperate phages could help *P. aeruginosa* select for bacterial characteristics that favor persistence of bacteria in the lung (Latino et al., 2014; Tariq et al., 2019). Thus, the *bci* gene may help clinical isolates of *P. aeruginosa* to survive in lung infections, increasing their chance of being infected by temperate phages.

In this research we identified two new prophages, Pf8 and AUS531phi, present in clinical *P. aeruginosa* strains of the CC274 clone, which cause infections in CF patients. Further research is required to determine the role of Pf8 inovirus bacteriophages (filamentous prophages) and their putative toxin/antitoxin system in chronic lung infections by *P. aeruginosa*. Also, we describe a new gene, *bci* (present in prophage AUS531phi), which is involved in regulating the bacterial QS system and favoring the infective capacity of the strain and therefore favoring the presence of this phage in the CF CC274 clone characterized by a low presence of prophages.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, (https://www.ncbi.nlm.nih.gov/nuccore/MN710383). https://www.ncbi.nlm.nih.gov/genbank/, (https://www.ncbi.nlm.nih.gov/nuccore/MN585195).

ETHICS STATEMENT

This study uses strains obtained from the work titled Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection from PlosOne 2013 (PMID: 23951065) and Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone published in Scientific Report 2017 (PMID: 28717172). Research Committee from A Coruña Hospital (Spain) which president is Maria Tomas, confirm it did not require the study to be reviewed or approved by an Ethics Committee because this collection of strains was previously published and in this work is not included clinical data from patients.

AUTHOR CONTRIBUTIONS

AA, LB, CL-C, RT, LF-G, IB, MP-A, OP, and ML conducted the experiments, analyzed the results, and wrote the manuscript. RC and TK revised the results. GB and AO revised the manuscript. MT obtained the research funding, directed the experiments, and supervised the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.556706/full#supplementary-material

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DISCUSSION

In the last decades, antimicrobial resistance has become a serious threat to global public health through years of developing versatile defence mechanisms against the most clinically relevant antibiotic agents (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance). Discovering and developing novel antimicrobial agents as an alternative to antibiotics are two relevant goals in microbiology research. The use of bacteriophages has emerged as one of the most promising strategies to combat multi-drug resistant microorganisms, either using them as lytic phages, their lytic enzymes (endolysins) or antimicrobial peptides (266). Also, the combination of these strategies with the use of antibiotics potentiates the action of both antimicrobial agents through a synergistic effect; thus, phage therapy can directly lyse bacterial host cells and those bacterial populations which survive to the phage attack are re-sensitize to the antibiotic by selective pressure (267).

Moreover, it is important to know the mechanisms by which bacteria and phage interact to design an adequate therapy and also to anticipate the potential benefits that some prophages could confer to clinical strains (268), (269). It is known that temperate bacteriophages drive host genome evolution through horizontal gene transfer transporting genes involved in stress tolerance, antimicrobial resistance, metabolic pathways, biofilm formation, tolerance and persistence mechanisms, virulence or quorum sensing (270-274).

In the research developed in chapter I, in order to obtain a lytic phage with therapeutic potential against the MDR bacteria *A. baumannii*, we selected the lysogenic phage (Ab105-2phi), which has a wide distribution in a group of clinical strains of *A. baumannii* belonging to the ST2 and isolated in 2010 as our group determined in previous work (14).

The disadvantage of the use of lysogenic phages in therapy is that they can act as carriers of genes related to resistance to antibiotics, virulence or toxins is solved by the prophage genome analysis by WGS and bioinformatic tools, enabling us to identify and characterize prophage regions and study them as a potential phage therapy (275). The analysis of the genome of the prophage

Ab105-2phi did not show any evidence of the presence of resistance, virulence or toxic genes, so this lysogenic phage was mutated into the lytic Ab105-2phiΔCI, a phage potential use in phage therapy. The transformation of the lysogenic phage into a lytic one was based on the principle that the CI gene represses the *Cro* gene needed for the development of the lytic cycle. Therefore, the deletion of the CI gene implies that there is no inhibition of the *Cro* gene, thus maintaining the cell in a permanent lytic state (56, 276). The conversion of a lysogenic phage by deleting the CI repressor gene was previously described in *Salmonella enterica* bacteriophage SPN9CC and in the mycobacteriophage BPs33DHTH_HRM10, recently used in a phage cocktail to treat a patient with a disseminated drug-resistant *Mycobacterium abscessus* (79, 275, 277).

The deletion of the gene *CI* and the conversion of the lysogenic phage Ab105-2phi into the mutant lytic phage Ab105-2phiΔCi were confirmed by sequencing and by the behaviour of the infection curves done with each phage. Also, the recovery of the bacterial culture growth after 5 hours of infection indicates the emergence of phage resistant bacteria, which is a common event in the infection with one phage, as a result of the adaptation of the bacteria to the phages in the relationship of bacteria-phage in microbial communities, both in the laboratory and natural populations (200).

The antimicrobial activity of the phage Ab105-2phiΔCI was demonstrated with the infection curves and biofilm assay, but in order to reduce the emergence of phage resistants and increase its potential to be used in phage therapy, combination treatments of the phage with antibiotics were done. Dickey et al. and Jo et al. (278, 279) demonstrated that PAS is an effective method against the antibiotic and phage resistance in *S. aureus.* In fact, in *P. aeruginosa* phage and antibiotics combinations have been proved to be more successful than antibiotics alone in eliminating biofilm populations (146, 163). This strategy has been proved to combat multi-drug resistant bacteria because of its double action: the PAS effect is stronger in comparison with both methods by separate (146) and the two different selective pressures provided by both bacteria and phage restricts the emergence of resistance on both sides (80, 280). The frequency of emergency phage resistant

mutants was lower when the phage was combined with antibiotics, instead of the frequency of resistant mutants in presence of the phage alone (almost 2 log decrease). A synergistic effect was detected when the mutant phage Ab105-2phiΔCI was combined with meropenem and imipenem antibiotics, restoring the antibiotic sensitivity when the combination was done at a high phage MOI of 10 and a MIC of 1/4 antibiotic. As the host strain do not possesses beta-lactamases, the restoration of the antibiotic sensitivity, must be due because most phage receptors are membrane proteins of the Resistance-Nodulation-Division (RND) efflux pump which confers resistance to several antibiotic classes, so when the phage binds to its receptor protein blocks the efflux pump (143, 144). Therefore, the elimination of the resistant mutants turns the AUS105-2phiΔCI into potential usage as a therapeutic phage in combination with antibiotics.

In the survival assays with *G. mellonella*, the antimicrobial activity of the combination with carbapenem antibiotics and the mutant lytic phage was confirmed by the increase of the survival of the *G. mellonella* in those larvae treated with the combination, although the survival when the combination included MIC 32 µg/mL of meropenem was not statistically significantly higher. Consequently, the administration of a large number of Ab105-2phiΔCI would be necessary (*in vivo*) due to the high MIC of meropenem.

Phage therapy includes the infection treatments with phages but also with products derived from them, as are the lytic enzymes, named endolysins, which are able to hydrolase the peptidoglycan layer of the bacterial cell wall. The main advantage of the usage of endolysins against multi-drug microorganisms is that bacteria have not been demonstrated to develop any mechanism of resistance towards them (107). In chapter II, we isolated two endolysins, ElyA1 and ElyA2, from two prophages, Ab105-1phi and Ab105-2phi, presents in the genome of the clinical strain Ab105_GEIH-2010 (GenBank Umbrella Bioproject PRJNA422585) (14). The sequence analysis of both endolysins showed that both were lysozymes and belonged to the GH108-PG3 family, but despite their similarity, ElyA2 did not show any muralytic activity, on the contrary to ElyA1 which demonstrate muralytic activity against different strains and species of gram-negative bacteria

belonging to the ESKAPE group. The endolysin ElyA1 showed a wide host range against *A. baumannii* and *P. aeruginosa* clinical strains and a lesser host range against *K. pneumoniae* clinical strains. This host range specificity in endolysins depends on its cell wall binding domains (CBDs). The ability of binding to the cell wall of CBD is usually wider than the host ranges of the phage from which it is isolated and covers a great number of strains and species, usually the entire bacterial genus (107, 281-283). However, there is not a consensus of CBDs influence on the endolysin activity. Some lysins have been reported to require a CBD for complete lytic activity (284-288), whereas others are similar or even more active when the binding domain is removed (289-292).

Endolysins are generally more effective against gram-positive than gram-negative bacteria because the outer membrane of gram-negative bacteria provides a functional layer to defend the microorganisms against endolysin activity (86). Overcoming this protective barrier is the main challenge in designing endolysin-based treatments in gram-negative bacteria, and outer membrane destabilizing agents as EDTA can be used (293). Thus, in this case, the muralytic activity was measured in the presence of EDTA, but the use of this agent in therapy is only limited to topic treatment (293). In order to solve this problem, we developed a strategy combining the ElyA1 endolysin with an antibiotic capable of disturbing the stability and increasing the permeability of the outer membrane, the colistin, as its use in combination with endolysins was previously described in *A. baumannii* (294). The positively charged molecules of colistin interact with the negatively charged lipopolysaccharide of the outer membrane, causing damage and destabilization of it, even leading to cell death (295). The disadvantage of using colistin is related to its nephrotoxicity and/or neurotoxicity in clinical practice (295, 296). In our study, we observed *in vitro* and *in vivo* an important reduction in the colistin MIC when the combination was assayed in *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* in those strains which were sensitive to colistin. However, when the combination was tested in those strains which were resistant to colistin did not show any antimicrobial activity. This could be due to the type of colistin resistance mechanisms, like the

alterations to reduce the fluidity of lipopolysaccharide, reducing of porin pathways or capsule formation (297); all related to decreasing the outer membrane permeability and thus would impede the endolysin mechanism.

Finally, the efficiency of the combination of ElyA1 and colistin as a therapeutic strategy was demonstrated *in vivo* with a significant increase in the survival of the *G. mellonella* larvae. Also, two mice models were done, a skin infection model and a lung model, and in both cases, a significant reduction in the CFU counts was obtained when the mice were treated with this combination.

Phage therapy is a successful strategy to combat bacterial infections but the knowledge of the interactions between phages and their bacterial host is important in order to improve this therapy. The coexistence of bacteria and phages, also in form of prophages, drives coevolution in both organisms that determine its behaviour and relation, and therefore their response in a phage therapy (200).

One of the consequences of this interaction between them is the appearance of phage resistance mechanisms in bacteria. The importance of known the interactions of phage-bacteria and the emergence of avoidance bacterial mechanisms to the phage infection is crucial in order to use routinely phage therapy against MDR pathogens.

In chapter III we describe the phage resistant genes present in bacteria and their evolution in 18 metagenome sequences of clinical clone ST-2 of *A. baumannii* isolated from the “II Spanish Multicenter Study GEIH-REIPI *Acinetobacter baumannii* 2000-2010” (Umbrella Bioproject PRJNA422585) (251, 298). The technology of WGS has been used to support wet laboratory techniques to undercover the insights of the phage resistance and general phage-bacteria interactions in the last years (299-302). NGS has already been implemented in several routine microbiological healthcare laboratory workflows as a powerful tool to type pathogens, reveal the presence of antimicrobial resistance, viral-associated genes and discover relevant mechanisms for the bacterial phenotype (303, 304). In this scenario, we identified a wide variety of phage resistance genes in the ST-2 clone of the

two 2000 and 2010 collections of *A. baumannii* strains. We also found a slightly higher proportion of phage resistance genes in the 2010 collection in comparison to the 2000 collection. This could be explained through the constant evolution of bacteria due to the HGT (305). The anti-phage defence systems have been discovered to be regularly clustered in bacterial genomes (306). We detect a higher percentage of genes belonging to GI's in the 2010 strains in contrast to the 2000 strains, confirming the evolutive pressure of the bacteria to the phage infection (307).

Following the study of prophages and their interactions with bacteria, in the IV chapter, we perform a study of the prophages present in clinical strains of the CF clone CC274 of *P. aeruginosa*, that belongs to the high-risk clone ST-274, which is one of the most widespread clones in clinical populations (308, 309). In the last few years, whole-genome sequencing has been turned into a useful tool for diagnosis and clinical research (310). Combined with a wide number of bioinformatic tools, the search for prophages and prophage-related elements should be a very profitable tool to depict and predict important factors for clinical strains such as genomic diversity, phylogeny, resistance mechanisms or virulence factors (269, 311, 312). In a study done in CF patients infected by P. *aeruginosa* Liverpool epidemic strain (LES) James et al., demonstrated that temperate prophages regulate bacterial density *in vivo* (313). Moreover, in these LES strains, competitive fitness has been proved to be influenced by temperate phages killing phage-susceptible competitors (314). Temperate phages LESφ2 and LESφ4 have been proved to have a role in the host fitness advantage and polylysogeny in these strains (315).

In the *P. aeruginosa* CC274 clone, we identified 4 complete prophage regions in the 24 isolates: 3 of them belonged to the filamentous phage genus of Inovirus and the other one to Siphovirus. Prophages have been demonstrated to allow the host survival in diverse environmental conditions during infection and colonization, also developing a role in the evolution of different bacterial pathogens (206, 268). They usually contain genes that confer themselves a selective advantage in a bacterial community (e.g. immunity or anti-CRISPR genes), and genes that confer an advantage to its

host and therefore to the phage, these are genes that involved bacterial mechanisms (e.g. bacterial persistence or biofilm formation) and/or virulence-related genes (316). Within these selective advantages, one disadvantage to consider related to the phage therapy by lytic phages is the mechanisms of immunity against secondary phage infections (or superinfection exclusion) conferred by the prophages (271).

In *P. aeruginosa*, filamentous phages have been demonstrated to influence the pathogenicity infection in patients with cystic fibrosis, even they were founded in clinical strains with high resistance to antibiotics (317). Homologues of the filamentous phage Pf4 are widespread through *P. aeruginosa* isolates. They have been associated with superinfective forms in mature biofilms, increasing bacterial virulence (318). Secor et al. demonstrated that filamentous phages help biofilm formation in CF isolates because they aggregate around the cell assembling a liquid crystalline structure (319). High concentrations of filamentous phage will lead to form a liquid crystal due to the alignment caused by steric forces between phage particles. This liquid crystalline biofilm matrix provides *P. aeruginosa* with higher transmissibility in CF patients allowing biofilms to better tolerate desiccation and also a protection against aminoglycoside antibiotics (265).

From the three Inovirus-type prophages identified in the CC274 clone, one was different to those previously described, as it contained two genes that provide a competitive advantage, a putative toxin/antitoxin (TA) system and a methyltransferase (Pf8_ST274-AUS411, Gb accession number: MN710383). TA systems function in prophages remain unclear, but they may give a selective advantage to prophages in maintaining bacterial genomes in host evolution at different environments, as they are usually found near mobile genetic elements responsible for much of the laterally transferred DNA in bacteria (320-322). Methyltransferases in bacteriophages are a defence mechanism against the restriction-modification (RM) gene complex, thus overcoming the bacterial phage-resistance system (323), conferring a selective advantage to prophages (324).

Bacteriophages and host interact in a close way, regulating different mechanisms from each other, thus, previous studies have shown a possible relationship between quorum sensing (QS) and bacteriophage. Høyland-Kroghsbo et al., demonstrated that the QS system participates in the regulation of the host phage susceptibility in *E. coli*, playing a key role in bacterium-phage coevolution in natural environments (254). LuxR-type receptors, which have been found encoded on phage genomes, bind to the AHL produced by the host bacterium, being involved in gene expression and regulation of the density status of their bacterial hosts (325). López et al. proved the parallel molecular evolution in clinical strains of *A. baumannii* of functional QS systems and the acquisition of prophages between 2000 and 2010 (326). Besides, lysogenic bacteriophages select QS-efficient *P. aeruginosa* hosts, applying a selective pressure in mixed populations with the goal of conserving the bacterial QS system (257). Also, when the QS stimulates the increase in the cell surface, the number and the density of the bacteriophage specific receptors are increased, and therefore so is the bacteriophage adsorption rate (327). We identified a Siphovirus, the prophage AUS531phi (Gb accession number MN585195) which contains the *bci* gene. We demonstrated the QS relationship with the *bci* gene checking its increased expression in presence of two QS inducers, as well as the repressive role of this gene in QS-related genes to favour the infection by AUS531phi temperate phage. Moreover, the infection with the phage AUS531phi with the *bci* gene increases three virulence factors controlled by QS in *P. aeruginosa*: swarming motility, biofilm and pyocyanin production, which can have a role in the survival of the bacteria under stress conditions.

Altogether, the presence of both types of phages in *P. aeruginosa* clinical isolates from CF patients may influence the survival rate in lung infections, improving their chance of being infected by temperate phages which confer them evolutionary benefits (328). It has been shown that pathogenic strains bear a larger proportion of phage-related genes than non-pathogenic strains (260). Whole-genome sequence data enable researchers the identification of prophages and cryptic prophage elements, thus exposing the wide range of phage-related factors which influence bacterial infection (54, 258, 259, 329).

These factors are crucial to the evolution, virulence and resistance of the bacterial hosts in clinical populations (206). Taking into consideration the prophage presence may be crucial in fighting pathogenic bacteria in clinical environments through diverse strategies. For example, it is important to identify the external genetic reservoirs that prophages are and what is their effect on the bacterial ecology. Another example, these prophages, in a lytic phage therapy context, could be the insertion of the *bci* gene to improve the infection in potential therapy phages.

In this thesis, prophages are showed as crucial in bacterial infections in a two-way manner: their capacity of infection and specificity in clinical strains make them an option to consider for phage therapy through lysogenic to lytic conversion and as a natural resource of endolysins. Furthermore, it is important to consider the phage resistance appearance in clinical strains and that they could be important elements to consider in clinical infections such as infections in CF patients, as well as their relationship with the QS system.

CONCLUSIONS

Chapter I

1. The *Acinetobacter baumannii* temperate phage Ab105-2phi was transformed into the lytic phage Ab105-2phiΔCI. The antimicrobial activity of this mutant lytic phage was confirmed against an *A. baumannii* culture and biofilm.
2. The strategy of combining lytic phage Ab105-2phiΔCI and carbapenem antibiotics (imipenem and meropenem) improve the antimicrobial activity of both components against clinical *A. baumannii* in two ways: reducing the phage-resistance and restoring the sensitivity to antibiotics.

Chapter II

1. ElyA1 is an endolysin belonging to the lysozyme family GH108-PG3, with a broad spectrum of muralytic activity against clinical strains of the bacterial pathogens: *A. baumannii*, *P. aeruginosa* and some clinical strains of *K. pneumoniae*. This makes this endolysin a good candidate to be used in antimicrobial therapy.
2. ElyA2 is an endolysin of the lysozyme family G108-PG3, highly similar to ElyA1 but no antimicrobial activity was detected in this case.
3. The combination of ElyA1 with colistin was shown to be a useful antimicrobial strategy both *in vitro* and *in vivo*, allowing the access of the endolysin to the peptidoglycan in gram-negatives and reducing the colistin MIC.

Chapter III

1. Phage-resistance genes in ST-2 *A. baumannii* clinical strains present a higher presence in the 2010 strains in comparison to 2000 strains. Furthermore, in the 2010 strains phage resistance genes exhibit a higher percentage in GI's thus confirming the evolutive pressure.

Chapter IV

1. Pf8 inovirus is a new type of filamentous phage that was founded in a clinical strain of *P. aeruginosa* from a cystic fibrosis patient. This

phage is characterized by the presence of a putative toxin/antitoxin system and a methyltransferase which may have a role in chronic lung infections by *P. aeruginosa*.

2. The siphovirus phage AUS531phi contains the *bci* gene, a gene that favours the infection of the phage and controls the QS of the strain AUS531 of *P. aeruginosa*.

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ANNEXES

Supplementary material chapter II

Strains		Ceftazidime[a]	Imipenem[a]	Meropenem[a]	Tobramycin[a]	Amikacin[a]	Ciprofloxacin[a]	Colistin[a]
A.baumannii	GMA001	>128	4	16	<2	<2	>64	<0.5
	PON001	>128	8	8	8	<2	>64	1
P.aeruginosa	AUS531	3	2	0.75	1	6	0.125	1
	AUS601	>256	>32	>32	24	>256	16	0.25
K.pneumoniae	KP16	>16	>16	>16	>8	>32	>2	1
	KP17	1	16	8	≤1	≤4	≤0.06	1

TABLE S1. [a] MIC concentrations (mg/L)

Supplementary material chapter III

SUPPLEMENTARY MATERIAL

	Ab33_ GEIH-2010	Ab49_ GEIH-2010	Ab54_ GEIH-2010	Ab76_ GEIH-2010	Ab103_ GEIH-2010	Ab104_ GEIH-2010	Ab105_ GEIH-2010	Ab121_ GEIH-2010	Ab122_ GEIH-2010	Ab155_ GEIH-2000	Ab158_ GEIH-2000	161_ GEIH-2000	Ab166_ GEIH-2000	Ab169_ GEIH-2000	Ab175_ GEIH-2000	Ab177_ GEIH-2000	Ab183_ GEIH-2000	Ab192_ GEIH-2000
ABI	11	9	11	12	11	11	12	12	10	11	8	11	5	11	11	9	11	11
TA	35	35	32	31	32	32	35	33	33	32	32	30	26	36	36	29	36	36
RM	50	54	46	48	53	53	40	53	46	36	44	48	40	42	42	28	42	42
CRISPR -CAS	1	0	1	0	2	2	0	1	1	0	0	1	0	0	0	0	0	0
NEW	75	69	68	59	64	63	59	61	62	57	60	58	54	63	67	52	66	63
Total genes	3988	3861	3946	3908	3922	3947	3923	3936	3875	3759	3898	3864	3674	3745	3785	2811	3899	3688

Table 1. Absolute number of each phage resistance genes group in 18 genomes of *A. baumannii* clinical strains.

	Ab33_ GEIH-2010	Ab49_ GEIH-2010	Ab54_ GEIH-2010	Ab76_ GEIH-2010	Ab103_ GEIH-2010	Ab104_ GEIH-2010	Ab105_ GEIH-2010	Ab121_ GEIH-2010	Ab122_ GEIH-2010	Ab155_ GEIH-2000	Ab158_ GEIH-2000	Ab161_ GEIH-2000	Ab166_ GEIH-2000	Ab169_ GEIH-2000	Ab175_ GEIH-2000	Ab177_ GEIH-2000	Ab183_ GEIH-2000	Ab192_ GEIH-2000
ABI	1	1	1	1	1	2	3	2	1	2	1	0	0	1	1	2	2	1
TA	5	6	4	2	3	5	7	4	3	4	5	5	0	5	10	0	4	2
RM	26	26	25	18	27	23	12	26	23	12	19	21	16	18	20	9	11	22
CRISPR -CAS	1	0	1	0	2	2	0	1	1	0	0	1	0	0	0	0	0	0
NEW	10	9	9	8	6	9	10	10	11	8	8	5	3	6	5	5	0	7
Total genes in GIS	43	42	40	29	39	41	32	43	39	26	33	32	19	30	36	17	25	33

Table 2. Absolute number of genes predicted

SUMMARY (In Spanish and Galician)

O número de infeccións nosocomiais está incrementando debido ao crecemento das bacterias con múltiple resistencia aos antibióticos, incrementando o tempo de estadía no hospital e a mortalidade. Dentro das familias que causan este tipo de infeccións encóntranse *Acinetobacter baumannii* e *Pseudomonas aeruginosa*.

Os fagos son a entidade biolóxica con maior presenza na Terra, mantendo un rol esencial preservando o equilibrio microbiolóxico. Frederick Twort foi a primeira persoa que os describiu en 1915 e Félix D'Hérelle o primeiro en observar o seu potencial terapéutico en 1917. Sen embargo, mentres que en occidente a opción para tratar infeccións causadas por bacterias foron os antibióticos, a terapia de fagos só utilizouse históricamente no leste de Europa, especialmente nos países que formaban parte da antiga URSS. Debido á emerxencia mundial que supoñen as bacterias multirresistentes, así como o estancamento ao crear novos antibióticos eficaces contra elas, a terapia de fagos propúxose como alternativa para tratar infecciósn agudas e crónicas.

Os fagos poden experimentar dous tipos de ciclos despois da infección: lítico e lisoxénico. No ciclo lítico, o fago utiliza a maquinaria celular para replicarse e poder saír lisando a célula. No lisoxénico, os fagos lisoxénicos ou temperados insertan o seu DNA no cromosoma hóspede gracias ás integrasas. Unha vez integrado no xenoma, o fago (coñecido como profago neste estado) pode permanecer integrado por xeracións, pero pode cambiar a ciclo lítico en calquer momento grazas a un proceso chamado indución, que pode ocorrer de forma espontánea ou debido a que a bacteria está sometida a condicións non favorables de estrés.

A terapia de fagos pode aplicarse de diferentes maneiras dependendo do tipo de infección e para poder aproveitar as súas vantaxes e superar as súas desventaxas. Os fagos utilizáronse solos ou en cócteles de fagos, tamén combinando unha terapia de fagos con antibióticos para evitar as limitacións da terapia de fagos como, por exemplo, a aparición de resistencias aos fagos. Además, conseguíronse aillar e utilizar endolisinas, que son as proteínas que utilizan os fagos para lisar e saír da bacteria, como terapia

antimicrobiana eficaz baixo certas condicións ambientais tanto en bacterias gram-positivas como en gram-negativas.

Además de ser utilizados coma terapia, o estudio de fagos no ambiente bacteriano é importante á hora de coñecer as interaccións que ten coa célula e as súas posteriores consecuencias. Unha delas, que tamén supón o principal problema para a utilización da terapia de fagos, é a aparición de variantes bacterianas resistentes aos fagos. As resistencias aos fagos poden darse de diferentes formas de adaptación, como poden ser a mutación dos receptores que os fagos utilizan para adherirse á celula ou ben a adquisición de mecanismos con diferentes formas de interrumpir a infección: inhiben a adsorción do fago, bloquean a inxección do DNA, cortan o DNA inxectado, inhiben a replicación, interfiren no empaquetamento das proteínas virais ou incluso provocan o suicidio celular para evitar a propagación de fago ás células adyacentes. Un dos sistemas máis característicos debido á súa inmunidade adaptativa é o sistema CRISPR-Cas.

O quórum sensing (QS) é un mecanismo polo cal a bacteria regula a súa expresión xenética en resposta á densidade celular. Demostrou ser un interesante obxectivo terapéutico en bacterias multirresistentes como, por exemplo, no bloqueo das súas sinais específicas para previr a formación de biofilm. A relación entre o QS e a infección por fagos estudiouse nestes últimos anos en diversos organismos como en *P. aeruginosa*.

Nesta tese doctoral estudiáronse os fagos lisoxénicos e a súa relación con bacterias multirresistentes, tanto as súas aplicacións potenciais como terapia transformando un fago lisoxénico nun lítico ou utilizando as súas endolisinas; como as súas interaccións coa bacteria, ben sea producindo a aparición de resistencias contra os fagos ou interactuando coa rede QS.

No capítulo 1 desenvolveuse unha estratexia que pode expandir a dispoñibilidade dos fagos utilizados na terapia de fagos obtendo un fago lítico mutado dun lisoxénico, ademais de caracterizar a súa actividade antimicrobiana tanto só como combinado sinerxísticamente con antibióticos. O fago lisoxénico Ab105-2phi, identificado na cepa da colección do “II Estudio Nacional de *A. baumannii* GEIH-REIPI 2000-2010” (Umbrella

Bioproject PRJNA422585) Ab105_GEIH-2010, foi transformado nun fago lítico delecionando o xene do represor CI, que regula a fase lisoxénica dos fagos. O fago resultante foi observado mediante microscopía electrónica de transmisión (TEM) mostrando a mesma estructura do tipo “Siphoviridae” como o fago sen a mutación. Tamén caracterizouse o seu rango de hóspede ou “host range”, que afectaba a un 25% das cepas testadas, así como a súa eficiencia de plaqueo ou “efficiency of plating”, que era a máis elevada para a cepa Ab177_GEIH-2000. Por iso, e además por non ter fagos completos no seu xenoma, a cepa Ab177_GEIH-2000 foi seleccionada para probar o tempo de adsorción do fago, que foi 12 minutos; calcular a curva de crecemento ou “one-step growth curve”, que revelou un periodo de latencia de 30 minutos e o número de virións producidos por célula ou “burst size”, que foi sobre 32 ± 2 UFP ou Unidade Formadora de Placas por célula infectada.

Unha vez caracterizado, estudiouse a actividade antimicrobiana do fago. A actividade contra biofilm mostrou una gran reducción na cantidade de biomasa producida pola célula. As curvas de infección obtidas co fago lisoxénico sen a mutación en comparación co fago mutado Ab105-2phiΔCI confirmaron a natureza lítica do fago mutado observando un decrecemento drástico nas células infectadas co fago mutado. Sen embargo, sí que se observou un recrecemento despois de 5 horas debido a aparición de variantes resistentes aos fagos. Por iso, redúxose o ratio de aparición de variantes case 1 log nas UFC ou Unidade Formadora de Colonias resistentes ao fago infectando co fago lítico mutado Ab105-2phiΔCI en combinación cos antibióticos meropenem, imipenem e doxiciclina. Posteriormente, desenvolvimos un ensaio de norte bacteriana en presencia do fago a tres diferentes MOI’s ou Multiplicidades de Infección (MOI= 0.1, 1 y 10) e tres antibióticos (meropenem, imipenem y doxiciclina) a dous diferentes CMI’s o Concentración Mínima Inhibitoria (CMI’s= 1/4 y 1/8). Observouse unha reducción no número de UFCs despois de 6 horas para todas as combinacións de meropenem e imipenem, pero non para doxiclina, co fago (entre 4 e 8 log de diferencia en UFC/mL), demostrando un efecto sinerxístico. Este efecto mantívose despois de 24 horas cando a

concentración dos antibióticos carbapenémicos foi de CMI=1/4 con MOI=10 no caso de meropenem y de MOI=1, 10 no caso de imipenem, cunha reducción de alrededor de 6 log UFC/mL en cada caso.

Finalmente, realizouse un ensaio de supervivencia en *Galleria mellonella*, onde o ratio de supervivencia foi maior cando as larvas que eran infectadas coa cepa Ab177_GEIH-2000 foron tratadas coa combinación de meropenem ou imipenem co fago Ab105-2phiΔCI. Sen embargo, só foi estadísticamente diferente ($p < 0.05$) coa combinación con imipenem.

No capítulo 2, identificáronse e caracterizaron dous endolisinas, ElyA1 e ElyA2 in vitro e in vivo contra cepas clínicas de patóxenos multirresistentes. Ademais, determinouse a actividade antimicrobiana dunha delas, ElyA1, en combinación co antibiótico colistina. Estas dúas endolisinas foron identificadas da secuencia xenómica dos fagos temperados en *A. baumannii* Ab105-1 phi e Ab105-2 phi, respectivamente. As secuencias dos respectivos profagos están presentes no xenoma da cepa Ab105_ GEIH-2010 illada na colección do "II Estudo Nacional de A. baumannii GEIH- REIPI 2000-2010" (Umbrella Bioproject PRJNA422585). Ambas endolisinas foron clasificadas como lisozimas (N- acetylmuramidasas) cun dominio C-terminal que se corresponde coa familia glicósido hidrolasa 108 e cun dominio de unión a peptidoglucano PG3 no N-terminal.

Despois de clonar e purificar as endolisinas, caracterizouse a súa actividade muralítica. No caso da endolisina ElyA1 foi necesaria a desestabilización da membrana externa con EDTA para que a endolisina puidese actuar sobre o seu obxectivo no peptidoglucano. A elevada actividade muralítica desta encima estableceuse a pH=8.5 e a 37º C. No caso de ElyA2 non se observou actividade, aínda que si efecto agregativo das células probablemente debido a un mecanismo celular de tensión.

Os ensaios de actividade antibacteriana de ElyA1 mostraron un amplo espectro de actividade: alta actividade contra 25 cepas clínicas de A. baumannii, actividade variable contra 25 cepas clínicas de *P. aeruginosa* e baixa actividade contra 13 de 17 cepas clínicas de *Klebsiella pneumoniae*. Como a endolisina ElyA1 non era capaz de superar a membrana externa, a

súa actividade antimicrobiana foi caracterizada en combinación con colistina, que é un antibiótico polipéptido que altera a membrana externa. Primeiro, determináronse as CMI' s naquelas cepas clínicas coa maior e menor susceptibilidade á endolisina. Despois, fíxose unha curva de mortalidade nas cepas en cuxa CMI a colistina sufriu polo menos unha redución 4 veces menor. Ao engadir ElyA1, a redución na MI de colistina en 4 de 6 cepas (2 de A. baumannii e unha de cada *P. aeruginosa* e *K. pneumoniae*). Na outra cepa analizada de *P. aeruginosa*, observouse unha redución na CMI 2 veces menor, mentres que na outra de *K. pneumoniae* non se detectou ningunha redución. Estes resultados indican unha acción sinerxística entre a colistina e a endolisina ElyA1 en todas as cepas sensibles a colistina.

Ademais, a actividade antimicrobiana desta combinación de ElyA1 e colistina ensaiouse in vivo en larvas de *Galleria mellonella* e modelos murinos de infección pulmonar e pel. Os resultados in vivo confirmaron os resultados obtidos in vitro: a supervivencia de *G. mellonella* era maior cando era tratada cunha combinación de colistina e ElyA1. No caso dos ratos, o reconto de bacteria foi significativamente reducido naqueles animais tratados coa combinación de ElyA1 e colistina en ambos os modelos.

No capítulo 3, estudáronse os sistemas de resistencia aos fagos en 18 xenomas de cepas clínicas, especialmente aqueles relacionados co sistema CRISPR- Cas, de 18 xenomas de cepas clínicas *A. baumannii* pertencentes ao clon ST-2 entre os anos 2000 e 2010 da colección "II Estudo Nacional de A. baumannii GEIH- REIPI 2000-2010" (Umbrella Bioproject PRJNA422585). 9 xenomas pertencían á colección do ano 2000 e 9 á do 2010.

Nestes xenomas detectouse *in silico* a presenza de xenes putativamente asociados a resistencia contra fagos relacionados con sistemas abortivos de infección (Abi), sistemas toxina-antitoxina (TA), sistemas restrición-modificación (R-M), sistema CRISPR-Cas e con outros sistemas recentemente caracterizados (ex. Zorya, Thoeris, Shedu ou Gabija, cuxa función en *A. baumannii* está aínda por determinar). Atopouse unha presenza lixeiramente maior destes xenes nas cepas do ano 2010 en

relación ás do 2000. Ademais, detectouse a súa presenza en illas xenómicas (IG' s) nun maior cociente nas do 2010 que nas do 2000.

Buscáronse os sistemas CRISPR-Cas seguindo o protocolo marcado por Shmakov con certas modificacións, debido á falta de resultados nos xenomas estudados utilizando as ferramentas de procura de sistemas CRISPR-Cas máis coñecidas. Atopáronse 40 arrays potenciais de CRISPR en 17 das 18 cepas. Ademais, localizáronse 705 proteínas cuxa función podería estar relacionada cos sistemas CRISPR- Cas.

No último e capítulo 4 da tese, analizáronse e caracterizáronse os profagos presentes no xenoma de 24 cepas clínicas de *P. aeruginosa* pertencentes ao clon internacional de fibrose quística (FQ) ST274-CC274. Identificáronse dous novos profagos, un inovirus e un siphovirus; este último cun xene, chamado *bci*, nunca antes descrito en fagos e que está relacionado coa habilidade do fago para infectar. Ademais, tamén se analizou a súa relación coa rede QS.

A análise xenómica dos 24 illados clínicos de *P. aeruginosa* revelaron a presenza de 4 profagos completos en 3 dos illados. 3 deles pertencían ao xénero de fagos filamentosos Inovirus, un grupo de fagos que promoven a formación de biofilm en *P. aeruginosa* nos pulmóns de pacientes con FQ. Dous dos Inovirus identificados eran similares aos Inovirus pf4 e pf5 de *P. aeruginosa*. O terceiro, presente na cepa AUS411, caracterizouse como un Inovirus novo. Por último, o cuarto era un profago do tipo Siphovirus presente na cepa clínica AUS531.

O novo fago Inovirus identificado no illado AUS411 designouse como pf8_ST274- AUS411 (pf8) (Gb: MN710383). A análise xenómica mostrou unha gran homoloxía nas proteínas do Inovirus pf4, pero tres proteínas eran características deste fago: un módulo putativo toxina-antitoxina e unha metiltransferasa. O Siphovirus identificado na cepa clínica AUS531 denominouse AUS531 phi (Gb: MN585195) e a análise da súa secuencia revelou a presenza dunha proteína denominada Bci (Bacteriophage Control Infection, denominada así polo control de infección do bacteriófago que exerce), que posúe un rol regulatorio do QS. Ambos fagos temperados, pf8

da cepa AUS411 e AUS531phi da cepa AUS531, observáronse mediante TEM confirmando a aparencia filamentosa do Inovirus pf8 e a aparencia cunha cola longa non contráctil e unha cápside icosaédrica do Siphovirus AUS531.

Analizouse o rol da proteína Bci na interacción do fago AUS531phi e a bacteria. Para iso, se delecionó o xene *bci* obtendo unha cepa mutante AUS531∆bci. A cepa foi cultivada en presenza de mitomicina e o profago, sen o xene *bci*, sendo inducida e extraendo así o fago mutante: AUS531 phiΔbci. A cepa mutada, AUS531Δbci infectouse co fago sen mutar e mutado para analizar o seu efecto na expresión dos xenes relacionados co QS, a súa curva de infección, motilidade e secreción de biofilm e piocianina. Os resultados demostraron que o xene *bci* incrementa a habilidade do bacteriófago para infectar *P. aeruginosa* regulando a rede QS, diminuíndo a expresión dos 4 xenes seleccionados (LasR, RhlR, QscR e PqsR) cando a cepa mutante AUS531Δbci foi infectada co fago sen mutar AUS531phi. A capacidade de infección do fago AUS531phi comparouse na curva de infección, sendo maior cando o fago levaba o xene *bci*, demostrando a relación deste xene coa regulación de diferentes factores de virulencia na bacteria, así como unha redución na motilidade e un incremento na produción de biofilm e secreción de piocianina.

Por tanto, pódese concluír que o xene *bci* presente no fago AUS531phi xoga un rol na habilidade infectiva deste fago regulando a rede QS e factores de virulencia (como piocianina e motilidade) do hóspede. Son necesarios máis estudos para elucidar o rol do fago pf8 do tipo Inovirus e os seus putativos sistema toxina/antitoxina e metiltransferasa.

Ao longo desta tese doutoral, os profagos mostráronse cruciais nas infeccións bacterianas dunha forma dobre: a súa capacidade de infección e especificidade en cepas clínicas fanos unha opción para considerar para a terapia de fagos converténdoos de lisoxénicos a líticos, como mostramos no capítulo 1, e sendo unha fonte natural de endolisinas, como mostramos no capítulo 2. Ademais, é importante considerar a resistencia aos fagos e a súa forma práctica de evitala, neste caso combinando ambos os tratamentos con

antibióticos. Tanto a procura de mecanismos de resistencias como a caracterización dos profagos xa presentes e a súa interacción coa propia bacteria na infección a través de sistemas como o QS, nos capítulos 3 e 4 respectivamente, son consideracións a ter en conta para poder ter unha terapia eficaz.

El número de infecciones nosocomiales está incrementando debido al crecimiento de las bacterias con múltiple resistencia a antibióticos, incrementando el tiempo de estancia en el hospital y la mortalidad. Dentro de las familias que causan este tipo de infecciones se encuentran *Acinetobacter baumannii* y *Pseudomonas aeruginosa*.

Los fagos son la entidad biológica con mayor presencia en la Tierra, manteniendo un rol esencial preservando el equilibrio microbiológico. Frederick Twort fue la primera persona que los describió en 1915 y Félix D'Hérelle el primero en observar su potencial terapéutico en 1917. Sin embargo, mientras que en occidente la opción para tratar infecciones causadas por bacterias fueron los antibióticos, la terapia de fagos solo se ha utilizado históricamente en el este de Europa, especialmente en los países que formaban parte de la antigua URSS. Debido a la emergencia mundial que suponen las bacterias multirresistentes, así como el estancamiento al crear nuevos antibióticos eficaces contra ellas, la terapia de fagos se ha propuesto como alternativa para tratar infecciones agudas y crónicas.

Los fagos pueden experimentar dos tipos de ciclos después de la infección: lítico y lisogénico. En el ciclo lítico, el fago utiliza la maquinaria celular para replicarse y poder salir lisando la célula. En el lisogénico, los fagos lisogénicos o temperados insertan su DNA en el cromosoma huésped gracias a las integrasas. Una vez integrado en el genoma, el fago (conocido como profago en este estado) puede permanecer integrado por generaciones, pero puede cambiar a ciclo lítico en cualquier momento gracias a un proceso llamado inducción, que puede ocurrir de forma espontánea o debido a que la bacteria está sometida a condiciones no favorables de estrés.

La terapia de fagos puede aplicarse de diferentes maneras dependiendo del tipo de infección y para poder aprovechar sus ventajas y superar sus desventajas. Los fagos se han utilizado solos o en cócteles de fagos, también combinando una terapia de fagos con antibióticos para evitar las limitaciones de la terapia de fagos como, por ejemplo, la aparición de resistencias a los fagos. Además, se han conseguido aislar y utilizar

endolisinas, que son las proteínas que utilizan los fagos para lisar y salir de la bacteria, como terapia antimicrobiana eficaz bajo ciertas condiciones ambientales tanto en bacterias gram-positivas como en gram-negativas.

Además de ser utilizados como terapia, el estudio de los fagos en el ambiente bacteriano es importante a la hora de conocer las interacciones que tiene con la célula y sus posteriores consecuencias. Una de ellas, que también supone el principal problema para la utilización de la terapia de fagos, es la aparición de variantes bacterianas resistentes a los fagos. Las resistencias a los fagos pueden darse de diferentes formas de adaptación, como pueden ser la mutación de los receptores que los fagos utilizan para adherirse a la célula o bien la adquisición de mecanismos con diferentes formas de interrumpir la infección: inhiben la adsorción del fago, bloquean la inyección del DNA, cortan el DNA inyectado, inhiben la replicación, interfieren en el empaquetamiento de las proteínas virales o incluso provocan el suicidio celular para evitar la propagación del fago a las células adyacentes. Uno de los sistemas más característicos debido a su inmunidad adaptativa es el sistema CRISPR-Cas.

El quórum sensing (QS) es un mecanismo por cual la bacteria regula su expresión genética en respuesta a la densidad celular. Ha demostrado ser un interesante objetivo terapéutico en bacterias multirresistentes como, por ejemplo, en el bloqueo de sus señales específicas para prevenir la formación de biofilm. La relación entre el QS y la infección por fagos ha sido estudiada estos últimos años en diversos organismos como en *P. aerugonisa*.

En esta tesis doctoral se han estudiado los fagos lisogénicos y su relación con bacterias multirresistentes, tanto sus aplicaciones potenciales como terapia transformando un fago lisogénico en uno lítico o utilizando sus endolisinas; como sus interacciones con la bacteria, bien sea produciendo la aparición de resistencias contra los fagos o interactuando con la red QS.

En el capítulo 1 se desarrolló una estrategia que puede expandir la disponibilidad de los fagos utilizados en la terapia de fagos obteniendo un fago lítico mutado de uno lisogénico, además de caracterizar su actividad antimicrobiana tanto solo como combinado sinergísticamente con

antibióticos. El fago lisogénico Ab105-2phi, identificado en la cepa de la colección del “II Estudio Nacional de *A. baumannii* GEIH-REIPI 2000-2010” (Umbrella Bioproject PRJNA422585) Ab105_GEIH-2010, fue transformado en un fago lítico delecionando el gen del represor CI, que regula la fase lisogénica de los fagos. El fago resultante fue observado mediante microscopía electrónica de transmisión (TEM) mostrando la misma estructura del tipo “Siphoviridae” como el fago sin la mutación. También se caracterizó su rango de huésped o “host range”, que afectaba a un 25% de las cepas testadas, así como su eficiencia de plaqueo o “efficiency of plating”, que era la más elevada para la cepa Ab177_GEIH-2000. Por ello, y además por no tener fagos completos en su genoma, la cepa Ab177_GEIH-2000 fue seleccionada para probar el tiempo de adsorción del fago, que fue 12 minutos; calcular la curva de crecimiento o “one-step growth curve”, que reveló un periodo de latencia de 30 minutos y el número de viriones producidos por célula o “burst size”, que fue sobre 32 ± 2 UFP o Unidad Formadora de Placas por célula infectada.

Una vez caracterizado, se estudió la actividad antimicrobiana del fago. La actividad contra biofilm mostró una gran reducción en la cantidad de biomasa producida por la célula. Las curvas de infección obtenidas con el fago lisogénico sin la mutación en comparación con el fago mutado Ab105-2phiΔCI confirmaron la naturaleza lítica del fago mutado observando un decrecimiento drástico en las células infectadas con el fago mutado. Sin embargo, sí que se observó un recrecimiento después de 5 horas debido a la aparición de variantes resistentes a los fagos. Por ello, se redujo el ratio de aparición de variantes casi 1 log en las UFC o Unidad Formadora de Colonias resistentes al fago infectando con el fago lítico mutado Ab105-2phiΔCI en combinación con los antibióticos meropenem, imipenem y doxiciclina. Posteriormente, desarrollamos un ensayo de muerte bacteriana en presencia del fago a tres diferentes MOI’s o Multiplicidades de Infección (MOI= 0.1, 1 y 10) y tres antibióticos (meropenem, imipenem y doxiciclina) a dos diferentes CMI’s o Concentración Mínima Inhibitoria (CMI’s= 1/4 y 1/8). Se observó una reducción en el número de UFCs después de 6 horas para todas las combinaciones de meropenem e imipenem, pero no para

doxiciclina, con el fago (entre 4 y 8 log de diferencia en UFC/mL), demostrando un efecto sinergístico. Este efecto se mantuvo después de 24 horas cuando la concentración de los antibióticos carbapenémicos fue de CMI=1/4 con MOI=10 en el caso de meropenem y de MOI=1, 10 en el caso de imipenem, con una reducción de alrededor de 6 log UFC/mL en cada caso.

Finalmente, se realizó un ensayo de supervivencia en *Galleria mellonella*, donde el ratio de supervivencia fue mayor cuando las larvas que eran infectadas con la cepa Ab177_GEIH-2000 fueron tratadas con la combinación de meropenem o imipenem con el fago Ab105-2phiΔCI. Sin embargo, solo fue estadísticamente diferente ($p < 0.05$) con la combinación con imipenem.

En el capítulo 2, se identificaron y caracterizaros dos endolisinas, ElyA1 y ElyA2 *in vitro* e *in vivo* contra cepas clínicas de patógenos multirresistentes. Además, se determinó la actividad antimicrobiana de una de ellas, ElyA1, en combinación con el antibiótico colistina. Estas dos endolisinas fueron identificadas de la secuencia genómica de los fagos atemperados en *A. baumannii* Ab105-1phi y Ab105-2phi, respectivamente. Las secuencias de los respectivos profagos están presentes en el genoma de la cepa Ab105_GEIH-2010 aislada en la colección del "II Estudio Nacional de *A. baumannii* GEIH-REIPI 2000-2010" (Umbrella Bioproject PRJNA422585). Ambas endolisinas fueron clasificadas como lisozimas (N-acetylmuramidasas) con un dominio C-terminal que se corresponde con la familia glicósido hidrolasa 108 y con un dominio de unión a peptidoglucano PG3 en el N-terminal.

Después de clonar y purificar las endolisinas, se caracterizó su actividad muralítica. En el caso de la endolisina ElyA1 fue necesaria la desestabilización de la membrana externa con EDTA para que la endolisina pudiese actuar sobre su objetivo en el peptidoglucano. La elevada actividad muralítica de esta enzima se estableció a pH=8.5 y a 37ºC. En el caso de ElyA2 no se observó actividad, aunque sí efecto agregativo de las células probablemente debido a un mecanismo celular de estrés.

Los ensayos de actividad antibacteriana de ElyA1 mostraron un amplio espectro de actividad: alta actividad contra 25 cepas clínicas de *A. baumannii*, actividad variable contra 25 cepas clínicas de *P. aeruginosa* y baja actividad contra 13 de 17 cepas clínicas de *Klebsiella pneumoniae*. Como la endolisina ElyA1 no era capaz de superar la membrana externa, su actividad antimicrobiana fue caracterizada en combinación con colistina, que es un antibiótico polipéptido que altera la membrana externa. Primero, se determinaron las CMI's en aquellas cepas clínicas con la mayor y menor susceptibilidad a la endolisina. Después, se hizo una curva de mortalidad en las cepas en cuya CMI a colistina sufrió al menos una reducción 4 veces menor. Al añadir ElyA1, la reducción en la CMI de colistina en 4 de 6 cepas (2 de *A. baumannii* y una de cada *P. aeruginosa* y *K. pneumoniae*). En la otra cepa analizada de *P. aeruginosa*, se observó una reducción en la CMI 2 veces menor, mientras que en la otra de *K. pneumoniae* no se detectó ninguna reducción. Estos resultados indican una acción sinergística entre la colistina y la endolisina ElyA1 en todas las cepas sensibles a colistina.

Además, la actividad antimicrobiana de esta combinación de ElyA1 y colistina se ensayó *in vivo* en larvas de *Galleria mellonella* y modelos murinos de infección pulmonar y piel. Los resultados *in vivo* confirmaron los resultados obtenidos *in vitro*: la supervivencia de *G. mellonella* era mayor cuando era tratada con una combinación de colistina y ElyA1. En el caso de los ratones, el recuento de bacteria fue significativamente reducido en aquellos animales tratados con la combinación de ElyA1 y colistina en ambos modelos.

En el capítulo 3, se estudiaron los sistemas de resistencia a los fagos en 18 genomas de cepas clínicas, especialmente aquéllos relacionados con el sistema CRISPR-Cas, de 18 genomas de cepas clínicas *A. baumannii* pertenecientes al clon ST-2 entre los años 2000 y 2010 de la colección "II Estudio Nacional de *A. baumannii* GEIH-REIPI 2000-2010" (Umbrella Bioproject PRJNA422585). 9 genomas pertenecían a la colección del año 2000 y 9 a la del 2010.

En estos genomas se detectó *in silico* la presencia de genes putativamente asociados a resistencia contra fagos relacionados con sistemas abortivos de infección (Abi), sistemas toxina-antitoxina (TA), sistemas restricción-modificación (R-M), sistema CRISPR-Cas y con otros sistemas recientemente caracterizados (ej. Zorya, Thoeris, Shedu o Gabija, cuya función en *A. baumannii* está todavía por determinar). Se encontró una presencia ligeramente mayor de estos genes en las cepas del año 2010 en relación a las del 2000. Además, se detectó su presencia en islas genómicas (IG's) en un mayor ratio en las del 2010 que en las del 2000.

Se buscaron los sistemas CRISPR-Cas siguiendo el protocolo marcado por Shmakov con ciertas modificaciones, debido a la falta de resultados en los genomas estudiados utilizando las herramientas de búsqueda de sistemas CRISPR-Cas más conocidas. Se encontraron 40 arrays potenciales de CRISPR en 17 de las 18 cepas. Además, se localizaron 705 proteínas cuya función podría estar relacionada con los sistemas CRISPR-Cas.

En el último y capítulo 4 de la tesis, se analizaron y caracterizaron los profagos presentes en el genoma de 24 cepas clínicas de *P. aeruginosa* pertenecientes al clon internacional de fibrosis quística (FQ) ST274-CC274. Se identificaron dos nuevos profagos, un inovirus y un siphovirus; este último con un gen, llamado *bci*, nunca antes descrito en fagos y que está relacionado con la habilidad del fago para infectar. Además, también se analizó su relación con la red quórum sensing (QS).

El análisis genómico de los 24 aislados clínicos de *P. aeruginosa* revelaron la presencia de 4 profagos completos en 3 de los aislados. 3 de ellos pertenecían al género de fagos filamentosos Inovirus, un grupo de fagos que promueven la formación de biofilm en *P. aeruginosa* en los pulmones de pacientes con FQ. Dos de los Inovirus identificados eran similares a los Inovirus pf4 y pf5 de *P. aeruginosa*. El tercero, presente en la cepa AUS411, se caracterizó como un Inovirus nuevo. Por último, el cuarto era un profago del tipo Siphovirus presente en la cepa clínica AUS531.

El nuevo fago Inovirus identificado en el aislado AUS411 se designó como pf8_ST274-AUS411 (pf8) (Gb: MN710383). El análisis genómico mostró una

gran homología en las proteínas del Inovirus pf4, pero tres proteínas eran características de este fago: un módulo putativo toxina-antitoxina y una metiltransferasa. El Siphovirus identificado en la cepa clínica AUS531 se denominó AUS531phi (Gb: MN585195) y el análisis de su secuencia reveló la presencia de una proteína denominada Bci (Bacteriophage Control Infection, denominada así por el control de infección del bacteriófago que ejerce), que posee un rol regulatorio del QS. Ambos fagos temperados, pf8 de la cepa AUS411 y AUS531phi de la cepa AUS531, se observaron mediante TEM confirmando la apariencia filamentosa del Inovirus pf8 y la apariencia con una cola larga no contráctil y una cápside icosaédrica del Siphovirus AUS531.

Se analizó el rol de la proteína Bci en la interacción del fago AUS531phi y la bacteria. Para ello, se delecionó el gen *bci* obteniendo una cepa mutante AUS531∆*bci*. La cepa fue cultivada en presencia de mitomicina y el profago, sin el gen *bci*, siendo inducida y extrayendo así el fago mutante: AUS531phiΔ*bci*. La cepa mutada, AUS531Δ*bci*, se infectó con el fago sin mutar y mutado para analizar su efecto en la expresión de los genes relacionados con el QS, su curva de infección, motilidad y secreción de biofilm y piocianina. Los resultados demostraron que el gen *bci* incrementa la habilidad del bacteriófago para infectar *P. aeruginosa* regulando la red QS, disminuyendo la expresión de los 4 genes seleccionados (LasR, RhlR, QscR y PqsR) cuando la cepa mutante AUS531Δ*bci* fue infectada con el fago sin mutar AUS531phi. La capacidad de infección del fago AUS531phi se comparó en la curva de infección, siendo mayor cuando el fago llevaba el gen *bci*, demostrando la relación de este gen con la regulación de diferentes factores de virulencia en la bacteria, así como una reducción en la motilidad y un incremento en la producción de biofilm y secreción de piocianina.

Por lo tanto, se puede concluir que el gen *bci* presente en el fago AUS531phi juega un rol en la habilidad infectiva de este fago regulando la red QS y factores de virulencia (como piocianina y motilidad) del huésped. Son necesarios más estudios para elucidar el rol del fago pf8 del tipo Inovirus y sus putativos sistema toxina/antitoxina y metiltransferasa.

A lo largo de esta tesis doctoral, los profagos se han mostrado como cruciales en las infecciones bacterianas de una forma doble: su capacidad de infección y especificidad en cepas clínicas los hacen una opción a considerar para la terapia de fagos convirtiéndolos de lisogénicos a líticos, como mostramos en el capítulo 1, y siendo una fuente natural de endolisinas, como mostramos en el capítulo 2. Además, es importante considerar la resistencia a los fagos y su forma práctica de evitarla, en este caso combinando ambos tratamientos con antibióticos. Tanto la búsqueda de mecanismos de resistencias como la caracterización de los profagos ya presentes y su interacción con la propia bacteria en la infección a través de sistemas como el QS, en los capítulos 3 y 4 respectivamente, son consideraciones a tener en cuenta para poder tener una terapia eficaz.

Curriculum vitae

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Age: 27

5 YEARS OF EXPERIENCE IN WET LAB AND DRY LAB

Predoctoral Researcher of the Group "Translational Microbiology" at INIBIC-CHUAC (A Coruña)
September 2017 – Present

Online stay learning bioinformatics in University of Leicester
October 2020 – February 2021

Vall d'Hebron Barcelona Hospital, Department of Microbiology (Barcelona)
December 2016 – June 2017
Practicum Student (Master's Internship)

Faculty of Medicine and Health Science (Reus)
April 2016 – June 2016
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San Joan de Reus University Hospital (Reus)
March 2016 – June 2016
Practicum Student (Degree's Internship)

Provincial Hospital of Pontevedra
July 2015
Practicum Student (Extracurricular Internship)

SKILLS

- Background in Microbiology, Biotechnology and Genomics fields
- Experience with laboratory techniques in microbiology and molecular biology

EDUCATION

PhD IN CELLULAR AND MOLECULAR BIOLOGY. Doctoral thesis about prophages, their potential applications and their interactions with multidrug resistant bacteria
September 2017 – Present (Final Stages. Thesis already deposited and waiting for tribunal response).
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MASTER'S DEGREE IN CLINICAL RESEARCH: CLINICAL MICROBIOLOGY
September 2016 – July 2017
University of Barcelona (UB), Autonomous University of Barcelona (UAB) and Pompeu Fabra University
MASTER'S DEGREE IN PHARMACEUTICAL BIOTECHNOLOGY (ERASMUS GRANT)
October 2015 – February 2016
Politechnika Wrocławska
BACHELOR'S DEGREE IN BIOTECHNOLOGY
September 2012 – July 2016
Rovira i Virgili University

SCIENTIFIC PUBLICATIONS

- Viral Related Tools against SARS-CoV-2. Fernandez-Garcia L et al. Viruses, 2020 Oct. PMID: 33081350
- Temperate bacteriophages (prophages) in *P. aeruginosa* isolates belonging to the international cystic fibrosis clone (CC274). Ambroa A. Front Microbiol. 2020 Sep. PMID: 33101229
- Mechanisms of Tolerance and Resistance to Chlorhexidine in Clinical Strains of Klebsiella pneumoniae Producers of Carbapenemase: Role of New Type II Toxin-Antitoxin System, PemIK. Bleriot I. Toxins (Basel), 2020 Sep. PMID: 32887507
- (p)ppGpp and its role in bacterial persistence: New challenges. Pacios O. Antimicrob Agents Chemoter. 2020 Jul. PMID: 32041137
- Genomic analysis of 40 prophages located in the genomes of 16 carbapenemase-producing clinical strains of *Klebsiella pneumoniae*. Bleriot I. Microb Genom. 2020 May. PMID: 32041137
- *In vitro* and *in vivo* efficacy of combinations of colistin and different endolysins against clinical strains of multi-drug resistant pathogens. L. Blasco. Sci Rep. 2020 Apr. PMID: 32041137
- Strategies to Combat Multidrug-Resistant and Persistent Infectious Diseases. O. Pacios et. al. Antibiotics (Basel). 2020 Feb. PMID: 32041137
- Combined Use of the Ab105-2φΔCILytic Mutant Phage and Different Antibiotics in Clinical Isolates of Multi-Resistant *Acinetobacter*

- Good performing on bioinformatic tasks, tools and databases. Next generation sequencing data analysis.
- Experience with Unix/Linux environments
- Knowledge in programming languages such as Python and Bash
- Communication and networking skills due to attendance at Congresses and Conferences
- Writing skills in publications of scientific peer-reviewed journals

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- Multiplex Real time PCR-shortTUB assay for detection of the *Mycobacterium tuberculosis* complex in smear-negative clinical samples with low mycobacterial loads. Alcaide F et al. J Clin Microbiol. 2019 Jul. PMID: 31189585
- Quorum and Light Signals Modulate Acetoin/Butanediol Catabolism in *Acinetobacter* spp. Tuttobene MR et al. Front Microbiol. 2019 Jun. PMID: 31281296
- Relationship Between Quorum Sensing and Secretion Systems. Pena RT et al. Front Microbiol. 2019 Jun. PMID: 31231316
- Relationship Between the Quorum Network (Sensing/Quenching) and Clinical Features of Pneumonia and Bacteraemia Caused by *Acinetobacter baumannii*. Fernandez-Garcia L. Front Microbiol. 2018 Dec. PMID: 30619184
- Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments. Trastoy R. Clin Microbiol Rev. 2018 Aug. PMID: 30068737
- Relationship between Tolerance and Persistence Mechanisms in *Acinetobacter baumannii* Strains with AbkAB Toxin-Antitoxin System. Fernandez-Garcia L. Antimicrob Agents Chemother. 2018 Apr. PMID: 29463538