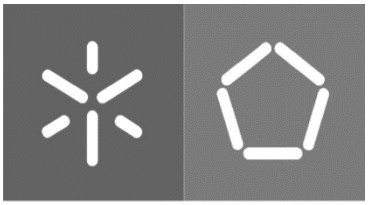
Universidade do Minho
Escola de Engenharia

Joana Teixeira da Costa Lucas

Novel Strategies to Combat Gram-Negative Bacterial Pathogens Using Bacteriophage Proteins

Outubro de 2015



Universidade do Minho
Escola de Engenharia

Joana Teixeira da Costa Lucas

Novel Strategies to Combat Gram-Negative Bacterial Pathogens Using Bacteriophage Proteins

Dissertação de Mestrado

Mestrado em Bioengenharia

Trabalho efetuado sob a orientação de

Doutor Sílvio B Santos

Outubro de 2015

DECLARAÇÃO

Nome: Joana Teixeira da Costa Lucas

Endereço eletrónico: joanatclucas@gmail.com Telefone: 912405399/227834320

Cartão do Cidadão: 14221126

Título da dissertação: Novel Strategies to Combat Gram-Negative Bacterial Pathogens Using Bacteriophage Proteins / Novas Estratégias para Combater Bactérias Patogénicas Gram-Negativos Usando Proteínas Bacteriofágicas

Orientadores:

Doutor Sílvio Roberto Branco dos Santos

Professora Doutora Joana Cecília Valente Rodrigues Azeredo

Ano de conclusão: 2015

Mestrado em Bioengenharia

Universidade do Minho, _____/_____/_____

Assinatura:

ACKNOWLEDGMENTS

"Agradeço aos meus orientadores, Dr. Sílvio Santos e Prof.ª Dra. Joana Azeredo, por me terem proporcionado a realização deste projecto. Um especial agradecimento ao Dr. Sílvio Santos por todo o incentivo, esclarecimentos e sugestões prestados durante a realização desta tese, e também, pelo à vontade e constante animação em laboratório, principalmente na presença do rádio."

"Aos restantes colegas do DEB, um muito obrigada por todos os momentos e camaradagem, em particular ao Carlos e ao Macedo, que tornaram os dias no laboratório mais divertidos, ao Zé por todas as explicações, histórias e conversas e à Cláudia, pela companhia no ginásio."

"Aos meus amigos, particularmente à Sílvia, à Inês e ao Manel por, quando em momentos de desespero, sempre me terem ignorado, levando-me a perceber que não havia motivo para tal."

"Aos meus pais e à minha irmã, devo-lhes a minha eterna gratidão, por sempre me terem apoiado e incentivado e nunca terem desistido de mim. Um obrigada a todos os familiares por toda a preocupação e interesse demonstrados."

"Obrigada Hugo, por me conheceres como mais ninguém conhece e por todos os momentos fantásticos que só tu me sabes proporcionar.

ABSTRACT

The infections caused by Gram-negative pathogens are a concern to global health, resulting in high rates of morbidity, mortality and high costs in health care. Once formed, biofilms cause reduction of host defenses and increase resistance to antimicrobial products 10 to 1000 times more than the cells in their planktonic state.

According to recent studies, in 2050, bacterial infections, associated with the growing resistance of pathogens to antimicrobial agents, will cause more than 300 million premature deaths and a loss of € 90 trillion to the global economy. At this scenario is extremely important the research and the development of new methodologies to eliminating bacteria and prevent or eradicate biofilm formation. The use of phage proteins presents several advantages as a potential alternative to antibiotics, since they are natural products with a low environmental impact and able to reduce aggregation and bacterial growth.

The aim of this project was to select and study the use of phage proteins to treat three Gram-negative bacteria: Pseudomonas, Salmonella and Escherichia. From the 18 proteins studied, 10 were expressed and these 4 had positive results. The holin from phage Lambda and the spanin Rz from phage T1 eliminated, with efficiency, planktonic cells of ATCC 10145 and protein T1_146 showed to be effective when applied to PAO1 planktonic cells. Biofilms of BL21 were effectively removed by Alginate Lyase.

The results showed the studied phage protein present high potential in the control of Gram-negative bacteria turning them an important alternative to antibiotics in the treatment of infection caused by these bacteria.

Keywords: Phage Proteins, Gram-negative Bacteria, Planktonic, Biofilms

RESUMO

As infeções causadas por bactérias patogénicas Gram-negativas são uma preocupação para a saúde global, resultando em taxas elevadas de morbidade, mortalidade e em altos custos em cuidados de saúde. Os biofilmes bacterianos provocam a redução das defesas do hospedeiro e o aumento da resistência a produtos antimicrobianos entre 10 a 1000 vezes mais que nas células no seu estado plantónico.

Segundo estudos recentes, em 2050, as infeções bacterianas, associadas à crescente resistência dos patogénicos aos agentes antimicrobianos, provocarão mais de 300 milhões de mortes prematuras e uma perda de €90 triliões para a economia global. Tendo em conta este cenário é da máxima importância a aposta e desenvolvimento de novas metodologias que permitam o controlo de bactérias e a formação de biofilmes. A utilização de proteínas fágicas apresenta várias vantagens, uma vez que são produtos naturais, com baixo impacto ambiental, capazes de reduzir a agregação e o crescimento bacteriano com baixa probabilidade de as bactérias desenvolverem resistência.

O objectivo deste projecto foi a seleção e estudo de proteínas (bacterio)fágicas para o controlo de três bactérias Gram-negativas com relevância clínica: Pseudomonas, Salmonella e Escherichia. De entre as 18 proteínas estudadas, 10 foram expressas e dessas, 4 apresentaram resultados positivos. A holina do fago Lambda e a proteína Rz do fago T1 eliminaram, com eficiência, as bactérias plantónicas de P. aeruginosa ATCC 10145 e a proteína T1_146 mostrou ser eficaz quando aplicada a células plantónicas de P. aeruginosa PAO1. Os biofilmes de E. coli BL21 foram os mais eficazmente removidos pela Alginato Liase.

Os resultados obtidos mostraram que as proteínas de origem fágica aqui estudadas apresentam um grande potencial no controlo de bactérias Gram-negativas constituindo uma alternativa aos antibióticos no tratamento de infecções provocadas por estas bactérias.

Palavras-chave: Proteínas Fágicas, Bactérias Gram-negativas, Plantónicas, Biofilmes

INDEX

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

AHLs – acyl homoserine lactones

AIs – autoinducers

AIPs – autoinducing peptides

APS – ammonium persulfate

BSA – bovine serum albumin

CFU – colony-forming unit

DNA – deoxyribonucleic acid

EDTA – ethylenediamine tetraacetic acid

EO – essential oils

EPS – extracellular polymeric substances

GI – gene identification

Gi – gastrointestinal

GN – Gram-negative

GP – Gram-positive

IM – inner membrane

Kan – kanamycin

LB – lysogeny broth

LPS – lipopolysaccharide

MCS – multiple cloning site

MW – molecular weight

O.D. – optical density

OM – outer membrane

ORF – open reading frame

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PES – polyethersulfone

PG – peptidoglycan

PGHs – peptidoglycan hydrolases

pH – potential hydrogen

pI – Isoelectric point

QS – quorum-sensing

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP – signal peptide

TAE – Tris-acetate-EDTA

TEMED – tetramethylethylenediamine

TM – transmembrane

TMH – transmembrane helices

UC – uncountable

1. State of the art

1.1 Gram-Negative Bacterial Pathogens

Bacteria are prokaryotes (unicellular organisms with no nuclear membrane, mitochondria, Golgi bodies, or endoplasmic reticulum) that reproduce by asexual division[1].

Bacteria, based on the Gram stain technique, are differentiated in two major categories: Gram-positive (GP), which remain stained as purple by crystal violet on washing and the Gram-negative (GN), which lose the crystal violet-iodine complex during decolorization with the alcohol rinse, but retain the counterstain safranin, appearing reddish or pink[1–4].

Gram-negative bacilli (GNB), that can be commensal organisms among normal intestinal flora, are responsible for numerous infections in community and nosocomial settings[5–7].

Structurally, the envelope of the GN organisms is free from teichoic or lipoteichoic acids and contains an inner or cytoplasmic membrane, the periplasm space and the outer membrane. The periplasm space contains peptidoglycan (PG) and comprises a variety of hydrolytic enzymes, which are important for the breakdown of large macromolecules during metabolism (phosphatases, lipases, nucleases, and carbohydrate-degrading enzymes) and may represent lytic virulence factors (collagenases, hyaluronidases, proteases and beta-lactamase). The outer membrane allows to distinguish the GN from Gram-positive bacteria and from spirochetes and is the major permeability barrier in GN bacteria. The outer surface of this membrane is composed predominantly by lipopolysaccharides (LPS), or endotoxins, and the inner leaflet of the membrane and the entire inner membrane are composed of phospholipids. Both bilayers can contain a range of different types of membrane proteins. In addition to conferring a GN staining characteristic, these structures are important virulence factors and partially determine antibiotic susceptibility[1,8,9]. The mentioned structures are presented on Figure 1.

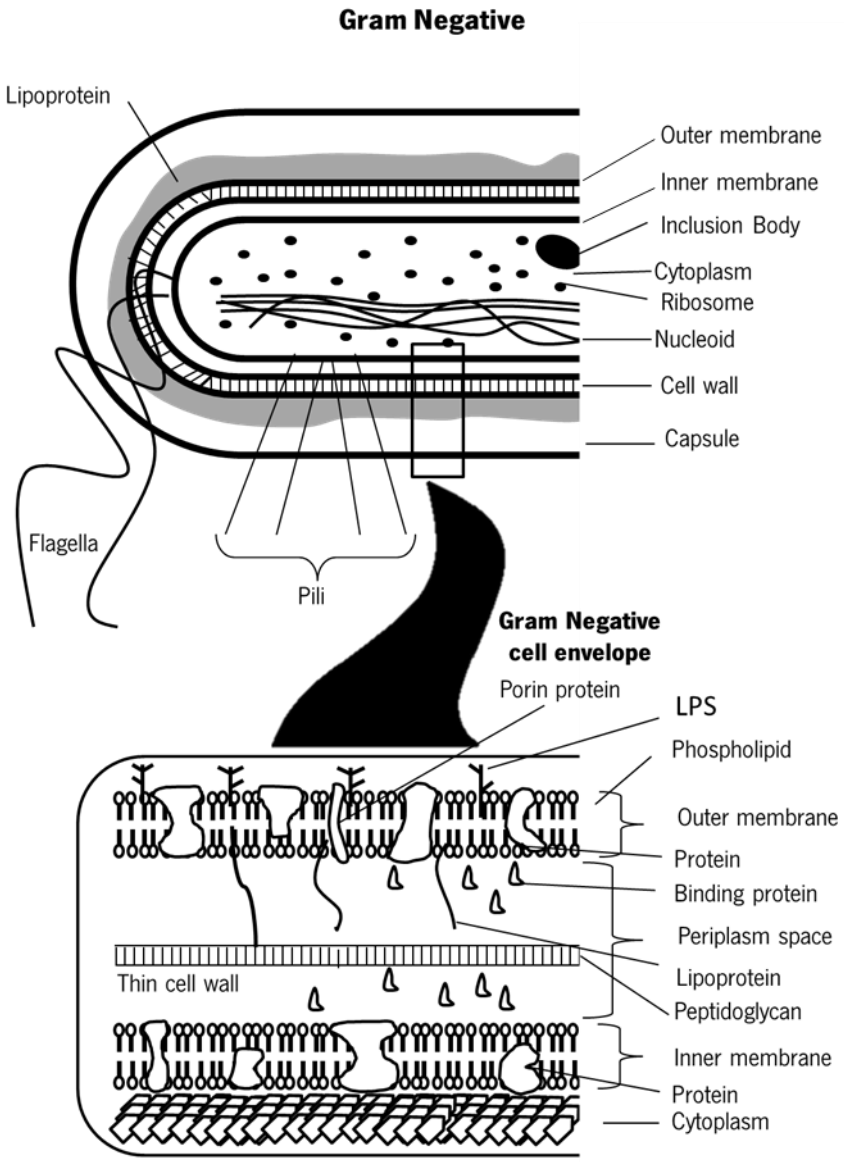


Figure 1- Components of the GN cell, detailing the structures of the envelope.

1.1.1 Planktonic Cells and Associated Problems

The human body provides the temperature, moisture, and resources necessary for bacterial growth. Moreover, many of the traits used by bacteria to enable them to invade the environment, to remain adhered or colonize, to gain access to resources (degradative enzymes), to escape clearance by host immune and nonimmune protective responses and the byproducts of bacterial growth are virulence factors, which enhance the ability of bacteria to cause disease[10].

The numerous microbes that colonize the human body, generally the gastrointestinal (Gi) tract, mouth, skin, and upper respiratory tract, compose the normal flora. Various microorganisms belonging to the

normal flora of hosts aid in important functions, such as: digestion of food, production of vitamins, protection from colonization with pathogenic microbes and activation of host innate and immune responses. An alteration of the normal flora, due to antibiotic treatment, diet, stress, and changes in the host response to the flora, can lead to inappropriate immune responses, causing inflammatory diseases[7].

Damages caused by bacteria are commonly due to the directly tissue damage, or to the release and dissemination of toxins through the blood. Those damages associated with the consequences of the innate and immune responses to the infection may result in a disease. Not all bacteria or bacterial infections cause disease; however, some always do it[1,11,12].

The importance of the affected organ and the extent of the damage caused by the bacterial infection gives an estimative of the seriousness of the disease. The gravity of the disease is also influenced by host factors, namely, congenital defects, immunodeficiency states and other disease-related conditions that increase the susceptibility to infection[1,13].

The number of bacteria, the capacity to spread, the potential to cause tissue damage and disease, and the host response determines the residence time of a bacterium in the organism[1,11].

1.1.1.1. Identification of Gram-negative Bacteria with Clinical Relevance

The GN bacteria are responsible for, among others, several respiratory tract infections, sexually transmitted diseases and Gi diseases. These microorganisms are the major cause of nosocomial (healthcare-associated) infections. The interactions of the host's susceptibility to infection, the organism's virulence potential, and the opportunity for interaction between host and organism mediate the development of an infection[1,11,12,14]. Some of the most important GN pathogens include:

- *Pseudomonas*, belonging to the *Pseudomonadaceae* family, an aquatic bacterium specie. As opportunistic pathogen, this organism can cause devastating chronic infections in compromised hosts and bacteraemia in immunocompromised hosts, representing an important source of nosocomial infections, wherein, the *P. aeruginosa* is the most important specie among more than 200[13,15–17]. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week[18–20].
- The family *Neisseriaceae* englobes three genera of medically important bacteria: *Neisseria*, *Eikenella*, and *Kingella*. Among the ten species of *Neisseria* found in humans, two are strictly human pathogens: *N. gonorrhoeae*, responsible for gonorrhea and *N. meningitides* that can colonize the nasopharynx of healthy people without producing disease or can cause community acquired meningitis, overwhelming

and rapidly fatal sepsis, or bronchopneumonia[21–23]. *Eikenella corrodens* and *Kingella kingae* are opportunistic pathogens that colonize the human oropharynx[24].

- The largest and most heterogeneous collection of medically important GN rods is the *Enterobacteriaceae* family. One third of all bacteremias, more than 70% of urinary tract infections (UTIs), and many intestinal infections are due to *Citrobacter freundii* and *C. koseri, Enterobacter aerogenes* and *E. cloacae, Escherichia coli, Klebsiella pneumonia* and *K. oxytoca, Morganella morganii, Proteus mirabilis, Salmonella enterica, Serratia marcescens, Shigella sonnei,* and *S. flexneri, Yersinia pestis, Y. enterocolitica* and *Y. pseudotuberculosis.* Those bacteria could be associated with human disease, with members of the normal commensal flora that can cause opportunistic infections or with commensal organisms that become pathogenic when they acquire virulence genes present on plasmids, bacteriophages, or pathogenicity islands[24].

- *Vibrio* and *Aeromonas*, belonging, respectively, to the families Vibrionaceae and Aeromonadaceae, are the second major group of GN rods. These organisms are found in water and are able to cause Gi diseases[25]. Within the genus *Vibrio* three species are particularly important human pathogens: *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The genus *Aeromonas* englobes 30 species, many of which associated with human disease[1,26].

- *Campylobacter* from the *Campylobacteraceae* family and *Helicobacter* belonging to the *Helicobacteraceae* family include the most important human pathogens[27,28].

- *Haemophilus* is a genus from the *Pasteurellaceae* family, responsible for a broad spectrum of diseases, with emphasis to the specie *H. influenzae* which commonly colonizes the human upper respiratory tract, although introduction of the *H. influenzae* type b vaccine has dramatically reduced the incidence of the disease particularly in the pediatric population[29].

- *Legionella pneumophila*, from *Legionellaceae* family, is an organism responsible for multiple epidemics and sporadic infections of pneumonia[30].

The most relevant diseases associated with the GN bacterial pathogens are listed in Table 1.

Table 1- Organisms and the clinical features associated[1]

Organism	Clinical Features
Pseudomonas aeruginosa	Pulmonary; primary skin and soft-tissue infection: burn wounds, folliculitis, osteochondritis; urinary tract infections; ear or eye infections; bacteremia; endocarditis
Neisseria gonorrhoeae	Gonorrhea, septic arthritis; pelvic inflammatory disease; perihepatitis; septicemia
Neisseria meningitides	Meningitis, septicemia (meningococcemia); pneumonia; arthritis; urethritis
Citrobacter	Meningitis; brain abscesses; hospital acquired infections
Enterobacter	Hospital acquired infections
Morganella	Hospital acquired infections
Escherichia coli	Watery diarrhea; diarrhea with mucus; hemorrhagic colitis; vomiting; hemolytic uremic Syndrome; Cystitis; pyelonephritis; acute meningitis
Klebsiella pneumoniae	Pneumonia; urinary tract infections
Proteus mirabilis	Urinary tract infections, wound infections
Salmonella enterica	Diarrhea; enteric fever (serovar Typhi)
Serratia, Enterobacter	Pneumonia; urinary tract infections; wound infections
Shigella	Bacillary dysentery
Yersinia	Bubonic and pulmonary plague; gastroenteritis (acute watery diarrhea or chronic diarrhea); transfusion related sepsis; mesenteric lymph nodes and mimic acute appendicitis
Vibrio cholera	Severe watery diarrhea; septicemia
Vibrio parahaemolyticus	Water diarrhea; wound infection
Vibrio vulnificus	Wound infections; primary septicemia
Aeromonas	Wound infections; gastroenteritis
Campylobacter jejuni, C. coli, C. upsaliensis	Gastroenteritis
Campylobacter fetus	Septicemia; meningitis; gastroenteritis; spontaneous abortion
Helicobacter pylori	Gastritis, peptic, and duodenal ulcers; gastric adenocarcinoma

Haemophilus influenza	Meningitis, septicemia, cellulitis, epidlottis; otitis media, sinusitis, bronchitis, pneumonia
Legionella pneumophila	Legionnaires' disease (pneumonia); Pontiac fever (flulike illness)

1.1.2 Bacterial Biofilms

Biofilms are complex sessile bacterial communities embedded in a matrix of extracellular polymeric substances (EPS), such as proteins, nucleic acids and polysaccharides[31,32].

The microbial population comprising a biofilm can be made up of a single or multiple bacterial species[19,33,34].The structures formed by biofilms are not static and cells may detach, leading to dispersion of the biofilm and formation of new ones[35].

Once developed, biofilms are harder to be removed completely[36,37]. Sessile bacteria are less susceptible to host defenses and more resistant to antimicrobial products (10-1000 times) than planktonic forms. This fact can be explained by the differences between planktonic and sessile cells physiology, gene expression and morphology and the different conditions (gaseous, nutrient stratifications) that the cells are exposed[38–42].

Diseases related with biofilms are typically persistent infections characterized by slow development, an ability to resist host immune defenses and a transient response to antimicrobial therapy[43].

Biofilm formation by human bacterial pathogens on implanted medical devices causes major morbidity and mortality among patients, and leads to billions of dollars in healthcare cost[44]. It's estimated that over 80% of bacterial infections in human involve the formation of biofilms[45,46].

1.1.2.1. Biofilm formation

The process of biofilm formation occurs in response to environmental changes, and involves multiple regulatory networks, which translate signals to concerted gene expression changes thereby mediating the spatial and temporal reorganization of the bacterial cell[47]. Biofilm development is a dynamic and multicellular process with several stages (Figure 2)[48,49].

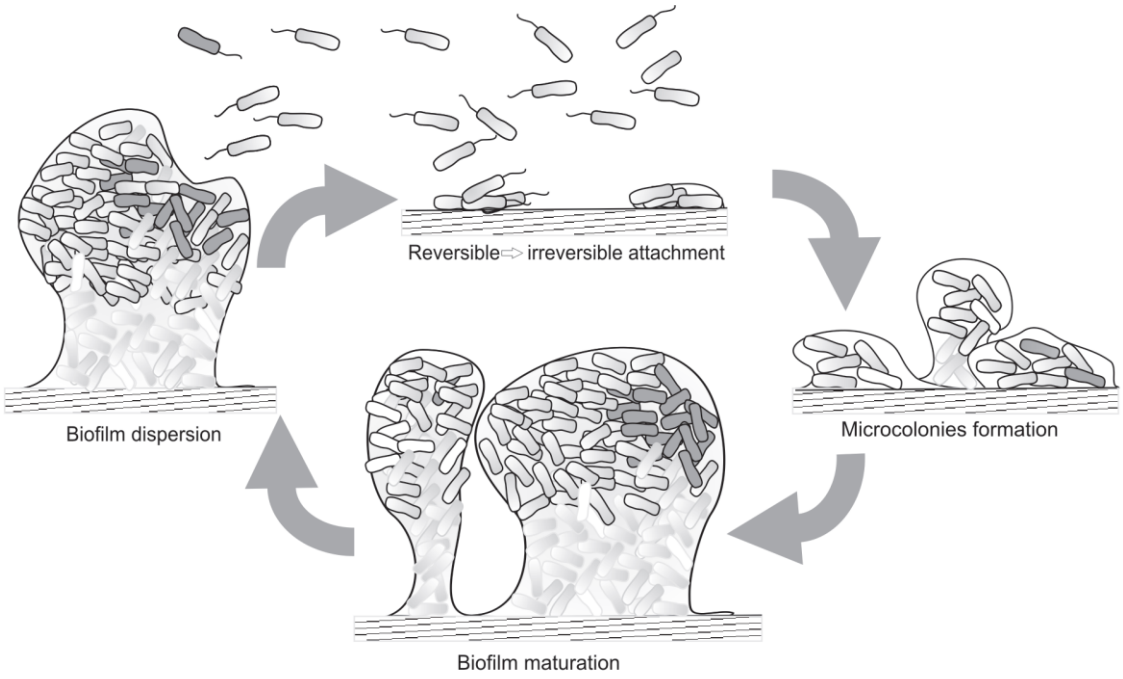


Figure 2- Stages of biofilm development[33].

Biofilm formation starts with the attachment of bacteria to a surface, a random process mediated by Brownian motion and gravitational forces, and influenced by surrounding hydrodynamic forces [50,51]. The structure of biofilms can be influenced by various bacterial activities such as cell growth or death, nutrient acquisition, waste product accumulation, motility mechanisms and exopolysaccharide synthesis[52,53].

Once microorganisms are attached to a surface, they are submitted to a series of changes to adapt at life on a surface, such as the expression of large quantities of exopolysaccharides that may protect the biofilm and lead to biocide resistance and the development of complex architectural features[54–58]. Bacteria with flagella have a competitive advantage to overcome hydrodynamic and repulsive forces. After intercepting the surface, additional extracellular adhesive appendages and secreted adhesion mediate the adherence. Initial attachment is dynamic and reversible, during which bacteria can detach

and rejoin the planktonic population if perturbed by hydrodynamic forces, repulsive forces, or in response to nutrient availability[59–62].

Surface contact leads to gene expression changes and up-regulating factors favoring sessility, such as those implicated in the formation of the extracellular matrix[51,63–66]. Within the mature biofilm there is a bustling community that actively exchanges and shares products that play a pivotal role in maintaining biofilm architecture and providing a favorable living environment for the resident bacteria. Despite this, in mature biofilms, dispersal becomes an option. Besides passive dispersal, brought about by shear stresses, bacteria have evolved ways to perceive environmental changes and evaluate whether it is still beneficial to reside within the biofilm or whether it is time to resume a planktonic lifestyle. Biofilm dispersal can be the result of several cues, such as alterations in nutrient availability, oxygen fluctuations and increase of toxic products, or other stress-inducing conditions. Once dispersed, bacteria can reinitiate the process of biofilm formation, on encountering a suitable environment[67,68].

Different classes of autoinducers (AI), signaling molecules, are responsible for mediation of the quorum-sensing (QS), a form of cell-to-cell interaction in bacteria, in response to the increase in cell density. GP bacteria employ autoinducing peptides while GN biofilms are mediated by acyl homoserine lactones (AHL)[69,70]. QS is an important event related to bacterial biofilm growth and differentiation[71–73].

In conclusion, biofilm formation is mediated by a combination of adhesion mechanisms, bacterial motility and QS phenomenon that protect the cells and increase its resistance to the antibiotics[74].

1.1.2.2.Identification of Gram-negative Biofilm with clinical relevance

Both GN and GP bacteria can form biofilms on indwelling medical devices such as catheters, mechanical heart valves and prosthetic joints[75]. *E. coli, K. pneumoniae, P. mirabilis,* and *P. aeruginosa* are responsible for the majority of the clinical cases involving biofilms from GN bacteria[75,76].

The main GN biofilms associated to human infections are listed in Table 2.

Table 2- Humans infections involving GN biofilms[77]

Infection or disease		Common biofilm bacterial species
Periodontitis		*Porphyromonas gingivalis*
Otitis media		Nontypable strains of *Haemophilus influenza*
Biliary tract infection		*E. coli*
Osteomyelitis		*P.* aeruginosa, *Acinetobacter spp.* and *Enterobacter spp.*
Bacterial prostatitis		*E. coli, Pseudomonas spp.* and *Klebsiella oxitoca*
Cystic fibrosis pneumonia		*P. aeruginosa* and *Burkholderia cepacia*
Meloidosis		*Pseudomonas pseudomallei*
Nosocomial infections	Intensive care unit pneumonia	*P. aeruginosa*, *Acinetobacter spp.*, and *Stenotrophomonas maltophilia*
	Contact lens	*P. aeruginosa*
	Urinary catheter cystisis	*E. coli*, *P. mirabilis*, *P. aeruginosa* and *Klebsiella spp.*
	Peritoneal dialysis peritonitis	*P. aeruginosa*, *E. coli*, and *Klebsiella spp.*
	Endotracheal tubes	*P. aeruginosa*, *K. pneumonia, P. mirabilis* and *E. coli*
	Biliary stent blockage	*E. coli* and *Klebsiella spp.*

It is estimated that 60% of nosocomial infections are derived from biofilm-related infections, many of which are caused by coagulase-negative staphylococci[78–81]. However, not only GP or GN bacteria form biofilms on indwelling medical devices, also yeasts cause this aggregate[76].

1.2 Clinical treatment of bacterial infection

Antibiotics are one of the most important forms of therapy for bacterial infections, caused by planktonic and sessile cells.

However, the efficiency of antibiotics is compromised by a growing number of antibiotic-resistant pathogens and by the failure on the discovery of new antibiotics to keep pace with the emergence of the pathogenic resistance, becoming one of the most serious and grievous challenge of the 21st century[82].

In the United States, according to the Centers for Disease Control and Prevention at least 23.000 people die and more than 2 million are sickened annually as a result of an infection with an antibiotic-resistant organism. According to a recent report from the United Kingdom, the antibiotic-resistance crisis, in 2050, will be responsible for a loss of up to €90 trillion to the global economy[83].

Multidrug-resistant GN organisms have emerged as a major threat to hospitalized patients and have been associated with mortality rates ranging from 30 to 70%[14].

Resistance to antibiotics in GN bacteria is due to various molecular and biochemical mechanisms, with special attention to the chromosomally encoded drug efflux mechanisms that are ubiquitous in these bacteria and play an important role in both intrinsic and acquired multidrug resistance of clinical relevance. The drugs can be extruded out of the cell by efflux transporters, which exist as either single-component pumps or multicomponent pumps (typically contain a pump, an OM channel protein (OMP), and an accessory membrane fusion protein (MFP)). The drug efflux, schematized on Figure 3, also interplays with other resistance mechanisms, such as the membrane permeability barrier, enzymatic inactivation or modification of drugs, and/or antibiotic target changes, increasing the levels of resistance[84].

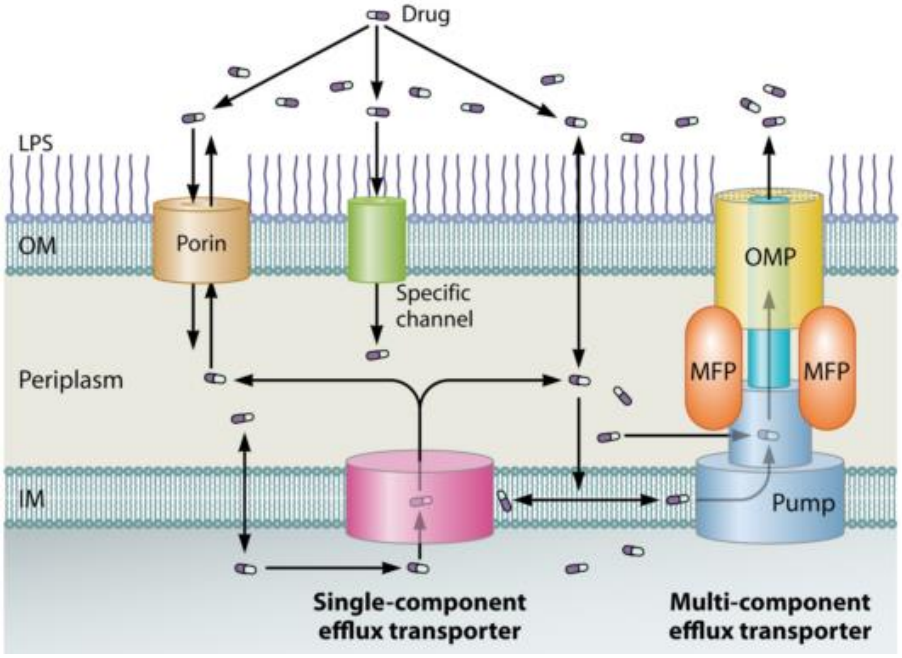


Figure 3- Drug efflux, the great responsible for antibiotic resistance in GN organisms[84]

β-lactam and aminoglycosides are two of the most widely available class of clinically important antibiotics for the treatment of various bacterial infections, whose effectiveness is now compromised[85].

Antipathogenic drugs are an alternative to the antibacterial drugs (*i.e.*, most traditional antibiotics) which target key regulatory bacterial systems that govern the expression of virulence factors, leading to the organism inability to establish successful infection[86].

Furanones, antipathogenics compounds similar to the natural AIs, inhibit the QS and presented positive results when tested to control *P. aeruginosa* infections in animal models. However, this form of therapy

is too reactive, and therefore presumably too toxic for the treatment of bacterial infections in humans. QS antagonist has a narrow spectrum, and, therefore, specific antagonists have to be developed for each organism targeted, allowing the attenuation of a single pathogenic organism living in a mixed population of normal bacterial flora, leaving the rest of the bacterial population unaffected. Although, some AHL signal molecules function as virulence factors, affecting the muscle tissue and tracheal gland cells[86].

The application of the drugs mentioned above, due to associated problems and disadvantages, is inappropriate and leads to the necessity to discovery and practice new forms of treatment to bacterial infections.

Dietary phytochemicals, such as essential oil (EO), phenolics, glucosinolates and their hydrolysis products, could represent a natural antimicrobial strategy with significant impact not only against planktonic bacteria but also on bacterial biofilm formation and development[87]. Some therapeutic hypotheses for sessile and planktonic bacteria are described below.

Planktonic

Borris and Sakanaka showed that catechins, a simple phenol present in the oolong green teas, inhibited, *in vitro*, the growth of *V. cholerae* and *Shigella spp*, and inactivated specific bacterial enzymes (toxin and glucosyltransferases) from the firsts[88,89].

Tannins, polymeric phenolic substances, present antimicrobial activity, responsible for the inactivation of microbial adhesions, enzymes and cell envelope transport proteins from several bacteria, including *E. coli*[90].

Copper, zinc, magnesium and especially silver and gold nanoparticles display antibacterial activity and are used for various healthcare. Rai and Chopra proved that Ag^+ (ionic silver) was active against *E. coli, S. aureus, Klebsiella sp.* and *Pseudomonas sp.*[91,92].

Thitiporn Anunthawan proposed that cationic peptides KT2 and RT2 bind to negatively-charged LPS to enable self-promoted uptake and, subsequently interact with cytoplasmic membrane phospholipids through their hydrophobic domains enabling translocation across the bacterial membrane, entry to the cells within minutes and liaison to DNA and other cytoplasmic membrane. These peptides, due to their antimicrobial and anti-biofilm activities may be an alternative to (or in conjunction with) conventional antibiotics to treat acute infections caused by planktonic bacteria and chronic biofilm-related infections[93].

Biofilms

Prevention of the biofilms' development is the first step for their control or eradication and the removal of the biofilm from contaminated device is an effective strategy for treating these infections[74,94]. However, prevention can not always be applied[95].

Coating the surface of biomaterial with bactericidal/bacteriostatic substances is one of the approaches to make it resistant to biofilms' formation[96].

The emergent resistance of biofilms to a variety of antimicrobial agents, including antibiotics, antiseptics and industrial biocides leads to the treatment failure without removing the bacterial infection[38,40].

Another parameter that can be used to remove biofilms is mechanical forces. This strategy is implemented with chemical agents, since they tend to leave the biofilm intact when no mechanical treatment is applied[37].

Different strategies to inhibit or disrupt biofilms at different stages of their development are summarized on Table 3.

Table 3- Examples of new strategies to inhibit or disrupt biofilms at different stages of their development[33]

Stage of biofilm development	Strategy to inhibit or disrupt biofilm formation
Reversible/irreversible attachment	Anti-adhesion agents, *e.g.* mannoside, pilicides, curlicides
	Antibiofilm polysaccharides, *e.g.* alginate, Pel, Psl
	Signal transduction interference, *e.g.* QS and two-component signaling
Microcolony formation and biofilm maturation	Silver nanoparticles
	Lytic phages, *e.g.* Escherichia coli T4 phage ATCC 11303-B4
	Enzymes degrading extracellular matrix, *e.g.* Dispersin B
	Antibiofilm polysaccharides
	Antimicrobial peptides, *e.g.* KT2, RT2
	Signal transduction interference, *e.g.* QS and two-component signaling
	Chelating agents, e.g. Ethylenediamine Tetraacetic Acid (EDTA)
Dispersion	c-di-GMP engineering to promote motility *vs.* sessility
	Introduction of dispersal signals, *e.g.* D-amino acids, norspermidine

Terpenoids are derived from terpenes (based on an isoprene structure) and due to their composition have a lot of biological functions and are applied as pharmaceuticals, fragrances and colorants. In 2005, Ren demonstrated that ursolic acid, a triterpenoid, inhibited biofilms of *E. coli* and *P. aeruginosa* and *Vibrio harveyi*[97,98].

Methyl eugenol, an EO with an aromatic ring, inhibit motility, QS, EPS production and biofilm formation by *P. aeruginosa*[99].

Borges demonstrated that gallic and ferulic acids (two phenolic compounds) have potential to inhibit bacterial motility, adhesion and to prevent and control biofilms of *P. aeruginosa*[100].

Davies proved that an unsaturated fatty acid, cis-2-decenoic acid, produced by *P. aeruginosa* in biofilm cultures, is responsible for inducing a dispersion response in biofilms formed by a range of GN bacteria, including *P. aeruginosa*[101].

Kolodkin-Gal concluded that the D-Amino acids, produced by many bacteria in stationary phase, prevent biofilm formation by *P. aeruginosa*[102].

The potential clinical value of antimicrobial agents that control and prevent *P. aeruginosa* infections by interruption of QS and inhibition of the transcription of biofilm-controlling genes or genes involved in cell attachment might also prove to be a successful strategy in inhibiting biofilm infections by interfering with various stages of biofilm maturation[95].

Various reports have suggested that macrolides act through effects on the immune system, modifying the inflammatory response to infection (as immunomodulatory), or through a direct effect in decreasing the virulence of *Pseudomonas*[40,95,103–111].

Despite the large numbers of approaches, neither can lead to completely inhibition or eradication of bacterial infection associated with planktonic or sessile cells, but only to the attenuation of their formation or effects. However, some of those therapies may become important in the control and decrease of these infections, being necessary additional research work and financial support.

The best results on the combat of problems related with bacteria are obtained when more than one strategy is implemented.

1.3 Bacteriophages

Bacteriophages (phages) are viruses that infect Bacteria and Archaea, also known as viruses of prokaryotes.

Approximately 96% of the 5500 reported phages have tailed morphology, which consist on a protein head, with a linear double-stranded DNA genome, enclosed by capsid, and a tail that promotes the phage attachment to the host cell and enable DNA injection[112].

In these phages, the first step in the infection process is the adsorption of the phage to the bacterial cell, mediated by the tail fibers or by some analogous structure that specifically recognize receptors on the bacterial cell such as proteins on the outer surface of the bacterium, LPS, pili and lipoprotein, in a reversible process[113]. Afterwards, one or more of the components of the base plate mediates the irreversible binding of the phage to the bacterium. This results in the contraction of the sheath and the hollow tail fiber is pushed through the bacterial envelope. Some phages have enzymes that digest various components of the bacterial envelope enabling the nucleic acid to pass into the bacterial cell. The rest of the phage remains on the outside of the bacterium. This process of infection can be made artificially even in a non-susceptible bacterium by injecting phage DNA through transfection[114].

The firsts steps on infection process are presented on Figure 4.

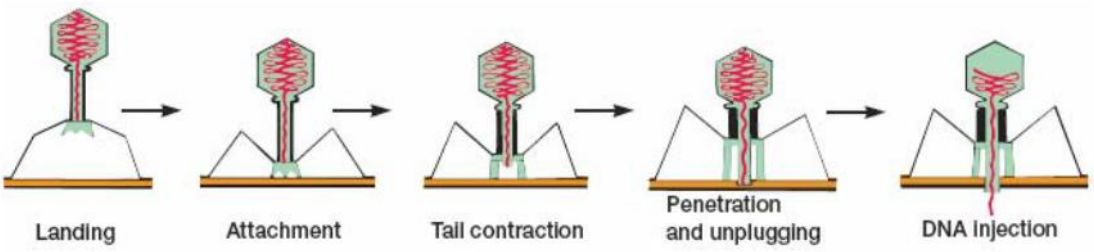


Figure 4- Adsorption and penetration of the phage to the bacterial cell[114]

The replication of the phages may occur in two different life cycles, lytic and lysogenic. Depending on the followed pathway, phages can be distinguished on strictly lytic and temperate.

Temperate phages may reproduce through the lytic or the lysogenic cycle. During the lysogenic pathway they are able to establish a persistent infection on the cell without killing it, since the phage DNA becomes integrated with the bacterial genome, originating the prophages which replicate synchronously along with the host chromosome for many generations, causing no harm to the host cell, as opposed to the lytic cycle[114–120].

The strictly lytic phages always follow the lytic pathway, infecting and inducing the bacterial cell lysis and resulting in the release of the progeny virions[114,116,117]. The liberated progeny phages are then ready to start another cycle by infecting new neighboring bacterial cells. The whole cycle can be completed in 20 to 40 minutes, depending on a variety of factors such as temperature, nutrients, light and other environmental forces, and during that time 50 to 200 viruses are released[115,121]. Once the phage

reproduction is much faster than typical bacterial reproduction, so entire colonies can be destroyed very quickly[116].

The life cycles are represented on Figure 5.

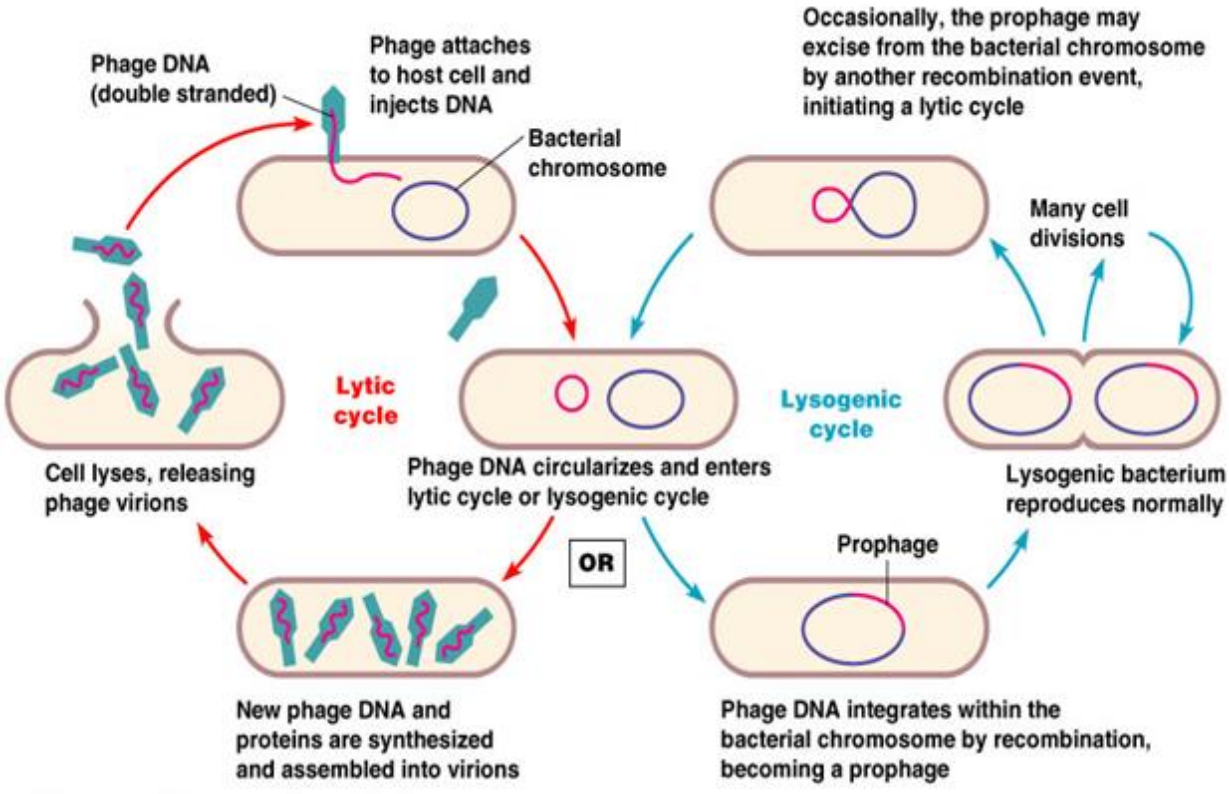


Figure 5-Lytic and lysogenic cycles. The replication of lytic bacteriophages follows the lytic cycle, while lysogenic bacteriophages may followed by lytic or lysogenic cycles[122]

1.3.1 Bacteriophage Therapy

The emergence of multiple drug-resistant bacteria has prompted interest in alternatives to conventional antimicrobials. Bacteriophage therapy, which consists on the use of bacteriophages as antimicrobial agents for the treatment of bacterial infections, has high potential as an alternative to antibiotics[123] due to abundance of phages in nature, their easy isolation, effectiveness to kill bacteria (even antibiotic-resistant bacteria at least in controlled laboratory experiments[113].

In opposite to antibiotics which usually target both pathogens and patients normal flora and travel throughout the body decreasing its concentration through time, bacteriophages are very specific to their hosts, minimizing the chance of secondary infections and tend to only minimally disrupt normal flora, replicating at the site of infection where they are mostly needed to lyse the pathogens.

It was demonstrated that the consumption of large amounts of phages has not led to any immunological complications, and topical application has not shown any adverse effects[124–127]. Contrarily, the antibiotics may cause allergies, sometimes even fatal anaphylactic reaction, and secondary infections[128].

Bacteriophages seem to be capable of disrupting bacterial biofilms, are environmentally friendly and are selected and isolated in a very rapid process[128,129], whereas the development of new antibiotic may take several years, may cost millions of dollars for clinical trials, and may also not be very cost effective [130].

Multiple experiments that focused on the therapeutic use of phages demonstrated an effective elimination of pathogenic bacteria from Gi diseases[131]. However, although the dynamics may differ, the evolution of bacterial resistance to a particular phage, just as to an antibiotic, is inevitable [132].

The predominance of lytic among temperate phages is an advantage to phage therapy, once that the lytic phages infect, lyse, and kill bacteria, until the infection is cleared or reduced to a level where the host immune system can effectively remove the remaining infection[133–135].

The temperate phages are unsuitable for phage therapy, as they do not lyse their host bacteria, and due to their ability to integrate into the genome of bacteria, may transduce the resistance genes from one bacterium to another, and thus paradoxically contribute to the spread of resistance between bacteria[113].

Bacteriophage therapy requires specific knowledge of phage biology. Some of the main problems arising from the use of phage therapy have recently been studied and are reported in Table 4[113,122,135].

Table 4- Some of the problems with early therapeutic phage research and the ways they have been addressed in more recent studies[122].

Problem	Comments
Narrow host range of phages	Because of the high specificity of phages, many negative results may have been obtained because of the failure to select phages lytic for the targeted bacterial species
Insufficient purity of phage preparations	Early therapeutic phages were crude lysates of host bacteria, and they contained numerous contaminants (including endotoxins) that may have counteracted the effect of phages
Poor stability and/or viability of phage preparations	Some commercial phage preparations were supplemented with mercurial or oxidizing agents or were heat treated to ensure bacterial sterility, many of these treatments also may have inactivated the phages, resulting in some ineffective phage preparations
Lack of understanding of the heterogeneity and mode of action of phages (*i.e.*, lytic or lysogenic phages)	Failure to differentiate between lytic and lysogenic phages may have resulted in the use of lysogenic phages, which are much less effective than lytic phages
Bacterial resistance to phages	This will unquestionably develop, although according to some authors the rate of developing resistance to phages is approximately 10-fold lower than that to antibiotics.

Additionally, there are some obstacles to phage therapy on biofilm infection, such as, difficulties of phage to penetrate the biofilm matrix and the presence of proteolytic enzymes and endoglucanases that can lead to inactivation of bacteriophages, the reduction of metabolic activity of biofilm cells and in the case of bacterial aggregate be formed by several species, these may bind and occlude phage receptors[136].

Although the use of phages to the treatment of bacterial infections present advantages relatively to antibiotics, the problems associated to their viral nature lead to the necessity of using another type of procedure, particularly proteins from phage origin.

1.3.2 Bacteriophage proteins in the treatment of Gram-negative Bacterial Pathogens

An interesting and promising phage-based therapeutic advance is centered in the use of phage encoded enzymes, produced actively during the lytic cycle, using the machinery of the host cell, which lysis the bacterial cell wall, from inside, allowing the release of phages[137–141].

Endolysins, also termed phage lysins or murein hydrolases, are phage-encoded peptidoglycan hydrolases (PGHs). These enzymes are employed by the majority of bacteriophages, at the end of their replication cycle, to enzymatically degrade the PG of the bacterial cell wall from within (by hydrolyzing the four major bonds), resulting in cell lysis and release of progeny virions. Lysis can be accomplished in two different ways: inhibition of PG synthesis by a single protein or enzymatic cleavage of PG by lysins or holin–lysin systems[142].

Historically, application of endolysins as antimicrobials has been limited to GP pathogens, due to the absence of an OM in cell wall, allowing the access of endolysins to the peptidoglycan from the outside (or from without). However, recent developments involving peptides with OM–disrupting properties fused to phage lysins have raised hopes of also tackling GN organisms with PGH enzymes[143–145]. In fact, Briers *et al.* recently reported the development of *Artilysins®*, a protein-engineered endolysin that passes through the OM and subsequently kill the cells through PG degradation and cell lysis[146]. Several published studies prove the influence and effectiveness of endolysins to lyse the cells, acting independently from others enzymes.

- The recombinant expression of endolysin Lys1521, from phage IAM 1521 of the *Bacillus amyloliquefaciens,* in *E. coli*, resulted in cell lysis, indicating that this enzyme is able to pass the cytoplasmic membrane independently. External application of Lys1521 on *E. coli* W3110 and *P. aeruginosa* reduced the number of cells with 98.75% and 99.78% in 10 min, respectively[147]

- LysAB2, an endolysin produced by *Acinetobacter baumannii* phage ϕAB2, when used in a high final concentration, show antibacterial activity against *A. baumannii*, *E. coli*, *Citrobacter freundii* and *S. enterica,* with a reduction between 67.5 and 99.9%, after 1 h of incubation[148].

- It was demonstrated that the P7 protein, from the *Pseudoalteromonas* PM2 phage, has bacteriolytic activity and is involved in the PG penetration process, causing a limited depolarization of the cytoplasmic membrane[149].

- A similar phenomenon was also observed with phages Φ13 (from *P. syringae)*, and ΦKMV and ΦKZ (infect *P. aeruginosa* strains) which contain a thermosensitive lytic enzyme involved in PG penetration[150–152].

- A study developed by Hanlon *et al.* found that a *P. aeruginosa* bacteriophage was able to penetrate the inner layers of the biofilm due to the reduction of the viscosity of the alginate matrix by enzymatic degradation[106].

Beside those enzymes, some phages encode a second PGH, the virion-associated peptidoglycan hydrolase (VAPGH). In contrast to endolysins, VAPGH degrade localized peptidoglycan during infection, being able to generate small holes through which the phage tail tube crosses the cell envelope to eject the phage genetic material at the beginning to the infection cycle[153,154].

- It was shown that the P5 protein from phage ϕ6 is active against *P. phaseolicola*, *P. aeruginosa*, *P. fluorescens* and *P. putida*, after destabilization of the outer membrane by incubation of the cells in chloroform-saturated buffer at room temperature. The VAPGH from phage ΦKZ, Gp181, also has a wide lytic spectrum including *P. aeruginosa*, *P. fluorescens* and *P. putida*[155].

During phage development in the infected bacterium, lysin accumulates in the cytoplasm in anticipation of phage maturation. Meanwhile, phage encoded small hydrophobic membrane proteins termed holins, which promote the membrane disruption, allowing that lysins access the PG, causing cell lysis and the release of progeny phage[142,156].

Holins control the activity of bacteriophage-encoded endolysins and the timing of lysis during bacteriophage infection by regulating the access of these enzymes to their substrate. This regulation can be achieved by one of two proposed mechanisms: through control of murein hydrolase transport across the cytoplasmic membrane or by mediating the release and activation of membrane-associated murein hydrolases[157–161].

The GN specific phages developed other protein responsible for crossing the outer membrane, whose encoding gene is typically located near the endolysin and holin genes, creating a three-component lysis cassette, the spanin[162]. Without spanin function, lysis is blocked and progeny virions are trapped in dead spherical cells, suggesting that the outer membrane has considerable tensile strength[163].

Biofilm polysaccharide protects the bacterial cells against the majority of bacteriophage. However, if a phage possesses a specific polysaccharide hydrolase, the depolymerase, it may be able to degrade macromolecule carbohydrates within extracellular polysaccharides and LPS surrounding bacterial cells and gain access to the bacterial surfaces. Consequently, it could cause biofilm disruption through cell infection and lysis, as well as EPS degradation[108,111,164].

Different polysaccharide depolymerases are known, such as, endorhamnosidases that hydrolyze outer membrane LPS of GN species, endolsialidases that degrade capsular polysaccharides of *E. coli*,

alginate lysases that degrade the capsules of *P. aeruginosa* and hyaluronidases that degrade capsules of streptococcal species[153].

In fact, bacteriophage-encoded proteins with antimicrobial activity and combinations between different enzymes and between enzymes and other biocide agents represent a promising alternative to antibiotics, as potential new antimicrobials against infectious diseases.

2. Materials and Methods

2.1 Cloning and Expression of Bacteriophage Proteins

The aim of this work was to express bacteriophage proteins alone and fused with Lysin gp146 and tested them on GN bacteria, on sessile and planktonic forms.

2.1.1 Bacteria, Plasmid, Phages and Growth Conditions

The bacteria strains used to test the proteins were *Escherichia coli* strains BL21 Gold (DE3) purchased from Epicurian Coli line of *Stratagene*; *E. coli OverExpress C43(DE3) from Lucingen, ArcticExpress (DE3) from Agilent Technologies, Salmonella* Enteritidis strains 1400, isolated by the group in the scope of the European project PhageVet-P[165]; and *P. aeruginosa* strains ATCC 10145 and PAO1[166].

All bacteria were grown at 37 ºC on Lysogeny Broth (LB Broth- *nzytech*), at 120 rpm.

The plasmid pET-28a acquired from *Novagen* was used to accomplish the desired constructions. The restriction map and Multiple Cloning Site (MCS) are presented in Annex I, Figure 12 and Figure 13, respectively.

The DNA (deoxyribonucleic acid) from phages lambda and T7 were purchased from *FRILABO*. The DNA from phages T1 and T4, acquired from *DSMZ*, were extracted from phage lysate using the kit NucleoSpin Tissue (250), from Macherey Nagel Bioanalysis- Fisher Scientific, according to the manufacturer's instructions. The phage PVP-SE1, isolated by the group in the scope of the European project PhageVet-P[165].

2.1.2 Phage DNA Extraction and Gene Amplification

The bacteriophage proteins tested in the treatment of biofilms and planktonic bacteria above mentioned are listed in Table 5. The enzyme Alginate Lyase, from *Sigma-Aldrich®* (Product Number: A1603) was also tested against biofilms.

Table 5- List of bacteriophage proteins to express

Host	Proteins type	Phage	Name	Gene Identification (GI) on NCBI
E. Coli	Holins	T1	holin T1	45686347
		T4	holin T4	9632830
		T7	holin T7	9627478
		lambda	holin Lambda	160380505
	Spanin	T1	RzT1	45686349
		lambda	Rz1Lambda	160338810
S. enteritidis	Lysin	PVP-SE1	gp146	363539667
	Colonic Acid degrading enzyme		gp49	363539570

A glycosyl hydrolase, dispersin B (GI: 30420959), was acquired from a previously plasmid construction and cloned into pET-28a, expressed and tested. The referred proteins (Table 5), excepting the gp49, were expressed and tested in fusion with gp146, originating: holin T1_146, holin T4_146, holin T7_146, holin Lambda_146, holin Lambda_L30_146, RzT1_146, Rz1Lambda_146 and dispersin B_146.

The amino acidic and nucleotide sequences from each protein were obtained and can be found in Annex II and Annex III (Table 21 and Table 22).

The sequences encoding each protein were amplified using genomic DNA as template and specific primers, whose quality and specificity was analyzed by the online computer tools *OligoAnalyzer 3.1* (https://eu.idtdna.com/calc/analyzer) and *BLAST* (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), respectively (Table 6).

Table 6- Primers sequences of the proteins and its characteristics

Protein	Primer	Sequence	GC Content (%)	T_m (°C)	PCR (bp)	Specificity (%)
holin T1/ **holin T1_146**	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGAAAGAGTTTTTAACGGCTGCTAC3'	40	55.8	246	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGTCTCCCCCTGATCTTAAGCG3'	55	56.1	1036	100
holin T4/ **holin T4_146**	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGGCAGCACCTAGAATATCATTTTC3'	38.5	55.4	687	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGTTTAGCCCTTCCTAATATTCTGGC3'	41.7	54.6	1477	100
holin T7/ **holin T7_146**	Forward (**NcoI**)	5'GGCAT**CCATGG**CAGTGCTATCATTAGACTTTAACAACGAATT3'	31	54.2	234	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGCTCCTTATTGGCTTTCTTCCAGTC3'	45.8	55.5	1024	100
holin Lambda/ **holin Lambda_146**	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGCCAGAAAAACATGACCTGTTG3'	41.7	56.4	348	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGTTGATTTCTACCATCTTCTACTCCG3'	40	54.1	1138	100

holin Lambda_L30_146	Forward (NcoI)	5'GGCATCCATGGCAATGCCAGAAAAACATGACCTGTTG3'	41.7	56.4	1176	100
	Reverse (SacI)	5'CGCGGGAGCTCGCCGCCCGCGGAGCCGGACGCCGCGCCCGCGGAGCCGCCCGCGCCGGAGGACGCGGAGCCCGCGCCCGCCGCGCCGGACGCGGAGCCCGGTTGATTTCTACCATCTTCTACTCCG3'	40	54.1		100
Rz T1/ Rz T1_146	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGAAACTTAAGAAAACGTGCATTGCAATT3'	30	57	432	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGCGCCTCCTTTTTTTCGTGCTTAC3'	47.8	57	1222	100
Rz1 Lambda/ Rz1 Lambda_146	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGCTAAAGCTGAAAATGATGCTCTG3'	38.5	56.1	213	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGGCCTCTCTCTGAGGGTGAAATA3'	50	55.9	1003	100

gp146	Forward (NcoI /SacI)	5'GGCATCCATGGCACGGGAGCTCATGAATG CTGCAATTGCGGAGATT3'	41.7	58	744	100
	Reverse (**XhoI**)	5'CGCCG**CTCGAG**CGAGGTTAGAACAGATTT TGCCTTTT3'	38.5	56		100
gp49	Forward (**NdeI**)	5'GGGCC**CATATG**ATGGCAGATCTATTACCT ACCGT3'	43.5	55.1	2161	100
	Reverse (**BamHI**)	5'GGGCC**GGATCC**TTAAGTCCTTTCGCTGTA TACTACG3'	40	54.2		100
dispersin B/ dispersin B_146	Forward (**NcoI**)	5'GGCAT**CCATGG**CAATGAACTGCTGCGTGA AGGG3'	55	58.1	1060	100
	Reverse (XhoI/NdeI)	5'CGCCGCTCGAGCATATGGATGGTCTCGTC CTTCAGGG3'	60	57.1	1850	100

●- Associated with fused Protein

●- Associated with singled Protein

●- Associated with both kind of Proteins

_- DNA annealing Sequence with target genes

Holin Lambda_146 and holin Lambda_L30_146 differs on the linker between the holin and gp146, wherein the second one has the linker identified by White *et al.*[167].

The PCR amplification of each CDS was made through the use of the proof reading polymerase *Phusion Green High-Fidelity DNA Polymerase* from *Thermo Scientific* (#F-534L), to a final volume of 100 µl, following the manufacturer instructions using the parameters presented on Table 7.

Table 7- Parameters used to the PCR

Step	Temperature (°C)	Time (sec)
1st	98	30
2nd	98	10
3th	55	30
4th	72	60
5th	Back to the 2nd step, 34 times	
6th	72	600
7th	4	∞

Once finished, 5 µl of each amplified sample and 5 µl of *1kb DNA Ladder* acquired from *New England BioLabs® Inc.* were loaded on 1% agarose gel electrophoresis to confirm gene amplification. Gels were visualized in a ChemiDoc™ XRS+ System with Image Lab™ Software *(Version 5.1 Bio-Rad Laboratories, Inc.)*[168].

The commercial kit *DNA, RNA and protein purification: PCR Clean up,* from *Nucleospin®* was used to clean up the PCR amplified genes. The concentration as well the 260/280 and 260/230 ratios of the purified genes were obtained by loading 1.5 µl of the cleaned up sample in *nanoDrop 1000 Spectrophotometer (Thermo SCIENTIFIC)*.

2.1.3 Gene Insertion in the pET-28a Cloning Vector

Taking into account the concentration, the genes and the plasmid were digested for 3 h, at 37 °C, following the amounts presented in Table 8, with the *FastDigest* enzymes (NcoI, XhoI, NdeI, BamHI and SacI) specified on Table 6 and purchased to *Thermo SCIENTIFIC*.

Table 8- Quantities of components used to make a digestion

Component	Gene	Plasmid
1st restriction enzyme	1 µl	1 µl
2nd restriction enzyme	1 µl	1 µl
10x FastDigest Buffer	2 µl	2 µl
DNA	Ca. 200 ng	Ca.1000 ng
H_2O	Complete until 20 µl	Complete until 20 µl

The *OPTIZYME™ Alkaline Phosphatase*, from *Fisher BioReagents®*, (Table 9) was added to the plasmid, to remove the phosphate groups from the cutting ends of the plasmid to avoid self-ligation. Incubation was carried for 1 h at 37 °C.

Table 9- Quantities of each element added to the plasmid

Component	Quantities (µl)
Plasmid Digestion (Table 8- Quantities of components used to make a digestionTable 8**)**	20
10x OPTIZYME AP Buffer	3
OPTIZYME Alkaline Phosphatase	1
H_2O	6
Final volume	30

Inactivation of the restriction enzymes and the alkaline phosphatase was carried at 80 °C for 15 min. The *T4 DNA ligase*, from *Thermo SCIENTIFIC,* was used as specified on Table 10 and incubated 1 h, at 22 °C to make the ligation between genes and plasmid.

Table 10- Quantities used to insert the genes into the plasmid vector

Component	Quantities
pET-28a DNA (linear vector)	20-100 ng
Protein DNA (insert)	1:1 to 5:1 molar ratio over vector
10X T4 DNA Ligase Buffer	2 µl
T4 DNA Ligase	0.5 µl
H_2O	Complete until 20 µl
Final volume	20 µl

To the fused proteins, the procedure for cloning into plasmid was the same, with the difference that the plasmid used was a construction of pET-28a with gp146.

2.1.4 Gene cloning in *E. coli*

The constructions obtained were used to transform chemically competent cells (previously prepared as specified in Annex IV) of *E. coli* CTOP10 through heat shock (Annex V).

The transformants were selected in a LB with kanamycin (kan) Petri dish, and 10 colonies were randomly chosen and incubated with 50 µl of LB and kan, for 1 h 30 min, at 37 °C.

A Colony PCR, using *DreamTaq Green DNA Polymerase* (*Thermo SCIENTIFIC* – #EP0713) and T7 primers was done to confirm transformation, following the conditions presented on Table 11.

Table 11- Parameters used to the Colony PCR

Step	Temperature (°C)	Time (sec)
1st	95	300
2nd	95	45
3th	45	30
4th	72	60
5th	Back to the 2nd step, 34 times	
6th	72	600
7th	4	∞

Colonies presenting a band with the correct size were recovered in solid LB with kan incubated overnight, at 37 °C.

The plasmids from each positively and grown clone were extracted by *DNA, RNA and protein purification: Plasmid kit,* from *Nucleospin®* and sent to sequencing to confirm the correct insertion of the genes.

Plasmids with a positive result were transferred to the expression strains, *E. coli* C43, *E. coli* BL21 and *E. coli* Artic Express as described in Annex V.

Three colonies of each transformation were submitted to colony PCR to confirm the correct transformation of the cells and a positive clone was selected for further preservation.

2.1.5 Protein expression

For a given protein, a pre-inoculum with the corresponding clone (bacteria with the correct plasmid) in 1 ml of LB with kan was incubated overnight, at 37 °C and 120 rpm.

At this phase, multiples approaches were tested to optimize the conditions of protein expression. To each essay, detailed on Table 12, inoculum of 100 ml of LB with kan was transferred to sterilized 250 ml Erlenmeyer flasks and 1 ml of the pre-inoculum was added, followed by the incubation, until the defined $O.D._{600}$ (Optical density). To induce the protein expression, 100 µl of 1M IPTG (1 µM final) were added to the inoculum and incubated overnight, at the conditions listed below.

Table 12- Different conditions tested to optimize the protein expression

Essay	Host	$O.D._{600}$	Before Induction		Induction with IPTG		Additional approaches
			°C	min	°C	rpm	
1	BL21 gold (DE3)	0.450	-	-	37	120	-
2	BL21 gold (DE3)	0.450	-	-	21	150	-
3	BL21 gold (DE3)	0.450	-	-	16	150	-
4	BL21 gold (DE3)	0.600	4	10	16	150	NaCl 2 M and Ethanol
5	BL21 gold (DE3)	0.900	4	10	16	150	1% glucose
6	BL21 gold (DE3)	0.600	4	10	16	150	1% glucose
7	C43 (DE3)	0.600	4	10	16	150	1% glucose
8	Artic express (DE3)	0.600	4	10	16	150	1% glucose
9	BL21 gold (DE3)	0.400	4	10	21	200	1% glucose
10	C43 (DE3)	0.400	4	10	21	200	1% glucose
11	Artic express (DE3)	0.400	4	10	21	200	1% glucose
12	C43 (DE3)	0.600	4	10	16	250	1% glucose and azide
13	BL21 gold (DE3)	0.600	4	10	16	250	1% glucose and azide
14	BL21 gold (DE3)	0.600	4	10	16	250	1% glucose

The NaCl 2 M and the ethanol were added to the culture, at the essay 4, after the $O.D._{600}$ reached 0.600. At the essays 5 to 14, 1% glucose was mixed with the inoculums in the Erlenmeyer flasks. The compound with azide, used on essays 12 and 13, was added to the Erlenmeyer before the induction with IPTG.

2.1.6 Protein purification

The bacterial suspension was centrifuged 15 min, at 4 °C and 9000 g. Afterwards, the supernatants were discarded (or recovered in the case of holins) for purification. Pellets with the cells were resuspended in 5 mL of lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, at pH 7.2) and cells were lysed through three cycles of freezing (-70 °C)/thawing (32 °C) and sonication (*Vibra-Cell™ VC505*, from *Sonics & Materials, INC*) for 5 minutes (30 sec ON, 30 sec OFF), at 40% amplitude. The resulting suspension was centrifuged for 15 min, at 4 °C and 9000 g.

The pellets with the cell debris (and cells not lysed) were resuspended in 10 ml of distilled water and the supernatants were recovered and filtered through a 0.22 µm PES (polyethersulfone) membrane filters (*Whatman*) to new Falcon tubes, identified and stored at 4 °C.

The filtered supernatant was purified using a gravitational column with 500 µl of Ni-NTA resin (*HisPur™ Ni-NTA Resin*, from *Thermo SCIENTIFIC™*). Briefly, columns were calibrated with 4 ml of callibration buffer (lysis buffer with 30 mM imidazole). The filtered supernatant was loaded into the column and the flow through (supernatants filtered and passed through the nickel columns) was collected and identified. The column was washed twice with 2 ml of wash buffer (lysis buffer with 50 mM imidazole) and collected. Elution was carried in two fractions of 200 µl + 500 µl using elution buffer (lysis buffer with 300 mM imidazole)

A 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis- Annex VI) was performed to confirm protein expression. To that, 10 µl of each sample and 10 µl of *SDS-PAGE Loading sample buffer 2x* (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) were pipetted. These mixtures were heated during 5 min, at 95 °C for denaturation and the total volume of samples and 5 µl of protein ladder were loaded onto the wells. The power supply was programmed for 2 h 30 min at 90 V. After that, the gels were stained with coomassie blue (40% methanol, 7% acetic acid, 52.975% water and 0.025% Coomassie Brilliant Blue R250), for 15 min and submerged in a distaining solution (40% methanol, 7% acetic acid and 53% water), twice, for 10 min. Revealed bands were analyzed for size and intensity to assess protein expression.

Samples with the desired expressed protein were dialyzed in 10 KD Amicon columns (*Amicon Ultra-0.5 mL Centrifugal Filters for DNA and Protein Purification and Concentration*, from *Merck Millipore*). To accomplish dialysis, the Amicon columns were previously submitted to passivation by adding 300 µl of 1% BSA (Bovine serum albumin) and incubating overnight at room temperature. The columns were centrifuged firstly, during 20 min, at 4 °C and 14000 g's, and secondly, inverted, for 2 min, at 4 °C and 1000 g's to remove all the BSA in solution.

The eluted protein was loaded in a column and centrifuged during 20 min at 4 °C 14000 g. The column was washed, twice, with 500 µl of PBS (Phosphate-buffered saline) and centrifuged for 20 min, at 4 °C and 14000 g's.

After centrifugation, 200 µl of PBS were added to the columns and incubated for 10 min at room temperature. Subsequently, the columns were inverted and centrifuged for 2 min at 1000 g's to recover the dialyzed protein (stored at 4 °C).

2.2 Evaluation of proteins activity against planktonic and sessile cells

Once dialyzed, the proteins were quantified by *Micro BCA™, Protein Assay* kit (*Thermo Scientific™ Pierce™*) and then the desired quantities were tested on planktonic and sessile cells of *P. aeruginosa* ATCC, *P. aeruginosa* PAO1, *S. enterica* S1400 and *E. coli* BL21.

2.2.1 Test on Planktonic Cells

To proceed to this essay, firstly pre-inoculums of each strain were prepared and incubated overnight at 37 °C and 120 rpm. The inoculums, consisting on 50 µl from pre-inoculums in 1000 µl of LB, were kept on incubator at 37 °C and 120 rpm until $O.D._{600}$ reached 0.300. At this moment, the inoculums were diluted in PBS, in a ratio of 1:100 (10 µl of inoculums in 1000 µl of PBS).

To 5 µl of each bacterium, previously diluted with PBS, and to a final volume of 20 µl, were mixted 5 µl of 1st protein/PBS, 5 µl of 2nd protein/EDTA/PBS and 5 µl of PBS as mentioned on Table 13, resulting in eleven different mixtures for each one of the four bacteria.

Table 13- Scheme of the proteins tested on planktonic cells.

Bacterium (5 µl)	1st Protein/PBS (5 µl)	2nd Protein/EDTA/PBS (5 µl)	PBS (5 µl)
P. aeruginosa ATCC 10145/ *P. aeruginosa* PAO1/ *S. enterica* S1400/ *E. coli* BL21	holin Lambda	146	PBS
	holin Lambda_supernatant	146	
	holin Lambda_146	PBS	
	holin Lambda_146 (supernatant)	PBS	
	holin T4	146	
	holin T4 (supernatant)	146	
	holin T1_146	PBS	
	holin T1_146 (supernatant)	PBS	
	Spanin RzT1	146	
	Lysin gp146	EDTA	
	(Control) PBS	PBS	

Once the contents were mixed, they were incubated for 2 h, at 37 °C and 120 rpm. Serial tenfold dilutions from $\log_{10}$=-1 to $\log_{10}$=-5 were realized to a final volume of 200 µl (180 µl of PBS and 20 µl of previous dilution). After that, drops of 10 µl from each dilution were pipetted to LB agar Petri dishes and incubated overnight at 37 °C for CFUs determination.

2.2.2 Test on Sessile Cells

Inoculums (10 µl of pre-inoculums with 1000 µl of LB, in a ratio of 1:100) were incubated at 37 °C and 120 rpm, until $O.D._{600}$ reached 0.600.

In a sterile 96-well plate, 100 µl of diluted inoculums were pipetted onto wells, to achieve a final concentration of $1*10^6$ CFU/ml. To avoid the evaporation of the liquid, 200 µl of sterile PBS were added to surrounding wells and the plate was sealed with parafilm and placed on packaging recipient previously filled with distilled water. The recipient was sealed and plates incubated for 48 h, at 37 °C and 120 rpm. The growth medium was renewed at 24 h and the 48 h biofilm was washed twice with 150 µl of PBS.

The proteins to be tested were added to the biofilms in the quantities presented on Table 14 and schematized on Figure 6.

Table 14- Proteins tested on biofilms

Enzyme	Volume of Enzyme (µl)	PBS (µl)
dispersin B	25	75
dispersin B_146	25	75
gp49	25	75
Alginate Lyase (10000 U/g)	100	-
Control	-	100

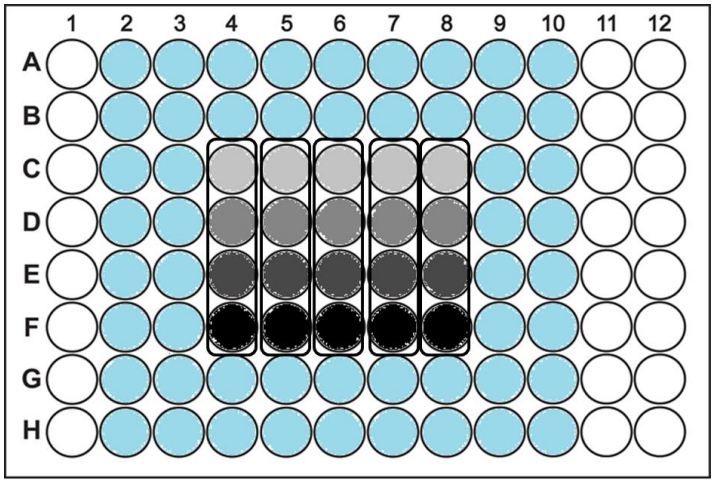


Figure 6- A 96 well plate scheme: wells on blue represent distilled water; grey wells schematize biofilms: line C - *P. aeruginosa* ATCC 10145, line D - *P. aeruginosa* PAO1, line E - *S. enterica* S1400 and line F - *E. coli* BL21. On columns are represented the proteins tested on biofilms: column 4 - dispersin B, column 5 - Dispersin B_146, column 6 - gp49, column 7 - Alginate Lyase and column 8 - the control.

Once added the proteins, the plate was incubated for 2 h, at 37 °C.

Cells in suspension were determined by removing, from each well, 20 µl of the suspension and pipetted to a new 96-well plate containing 180 µl of PBS, to proceed to successive dilutions from $\log_{10}$=-1 to $\log_{10}$=-5. The remaining 80 µl of the wells, where proteins were tested, were discarded and washed, twice, with 150 µl of PBS. After the washing, 100 µl of PBS were added to the wells and subjected to an ultrasonic bath (*Aquasonic Water-table Sonicator model 250 HT*, from *VWR Scientific*) for 30 min.

The wells were scraped and 20 µl from each were diluted on 180 µl of PBS, successively, from $\log_{10}$=-1 to $\log_{10}$=-5.

Droplets of 10 µl from each dilution were pipetted to LB plates. After overnight incubation at 37 °C, the CFU's were determined.

3. Results and Discussion

3.1 Bioinformatics Analysis

3.1.1 Prediction of transmembrane helices and signal peptides

The transmembrane helices (TMH) are membrane-spanning domains with a hydrogen-bonded helical configuration, including α, 310, and π-helices[169].

The prediction of TMH in integral membrane proteins is an important aspect of bioinformatics, since integral helical membrane proteins constitute an important subset of the proteins encoded by a genome, making up 20%–25% of the proteome and are crucial for many cellular processes, including signaling and transport processes[170]. They also influence the expression of the proteins, once are hydrophobic regions and may be associated with the toxicity of the proteins.

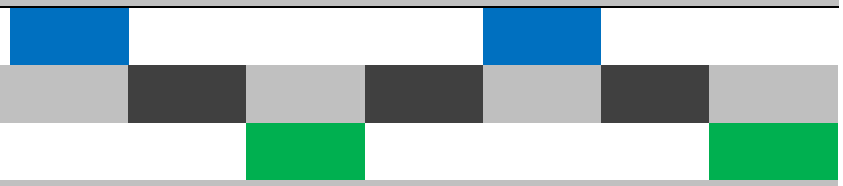
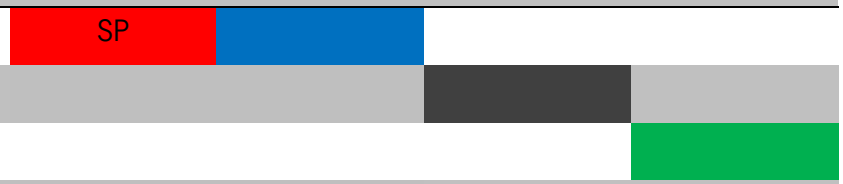
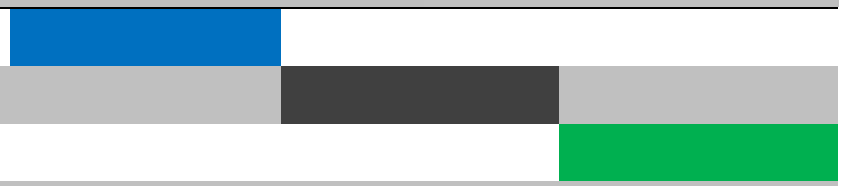
Secreted proteins and a majority of cell-surface proteins possess an N-terminal signal peptide (SP). The SP is typically between 15 and 40 amino acids long and is responsible to direct the proteins to its proper cellular location, being essential for protein secretion, being subsequently cleaved from the mature protein[171]. Problems related with the presence of SP may lead to the biologically inactivation of the proteins.

Phobius (http://phobius.sbc.su.se/) was used as the main prediction tool to the TMH and the SPs in proteins, backed up by *TMHMM Server v. 2.0* (http://www.cbs.dtu.dk/services/TMHMM/) and *SignalP 4.1 Server* (http://www.cbs.dtu.dk/services/SignalP-4.1/), respectively.

Based on hidden Markov model, these predictions are established on (1) the hydrophobicity analysis since that usually the TMH and SPs contain a stretch of hydrophobic amino acids and (2) the position of the first upstream charged residue. The different amino acid composition between cytoplasmic and periplasm or extracellular regions allow the prediction of the location of helices and their orientation with respect to the cell (pointing inside or outside the cell)[172,173].

The obtained results are presented on Table 15, and ANNEX VII (Figure 14 to Figure 22).

Table 15- Predicted TM topology and SP. CYT and green represent the residues present on cytoplasm; TMH (dark grey) illustrates the amino acids located within the membrane; NCYT, at blue, show the residues situated at periplasm or extracellular; and the NCYT, at red SP, represent the signal peptides. The AA No is the amino acid boundary between the different locations of each segment.

Proteins	Predicted TMHs	Predicted SP	Topology	
Dispersin B	0	0	AA No	343
gp49	0	0	AA No	712
gp146	0	0	AA No	236
holin Lambda	3	0	NCYT TMH CYT AA No	
holin T1	1	1	NCYT TMH CYT AA No	
holin T4	1	0	NCYT TMH CYT AA No	

holin T7	1	0	NCYT	
			TMH	
			CYT	
			AA No	1-36 37-55 56-67
Rz T1	1	1	NCYT	
			TMH	
			CYT	
			AA No	1-25 26-103 104-125 126-133
Rz1 Lambda	0	1	NCYT	
			AA No	1-22 23-60

3.1.2 Prediction of isoelectric point, molecular weight and proteins domains

The isoelectric point (pI) of a protein is defined as the pH at which the protein has a net charge of zero and, consequently, the amino acids do not migrate in an electric field. At potential hydrogen (pH) values below the pI, proteins carry a net positive charge and above those values, proteins carry a net negative charge. Solubility of hydrophobic proteins is lower at pI.

Molecular weight (MW) is a measure of the sum of the atomic weights of the atoms in a molecule. The prediction of MW contributes to a better evaluation of protein migration in SDS-PAGE.

To predict protein pI and MW the chosen tool was the *ExPASy* server (http://web.expasy.org/compute_pi/).

Protein signatures provide a description of a protein family, functional domains or conserved sites within related groups of proteins. These protein classifications are really important, once the residues in a family of proteins will be highly conserved if they are important for structure or function and determine the protein evolution. *InterPro* (http://www.ebi.ac.uk/interpro/) was used to provide an analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites. This resource combines predictive models from 11 different databases, making up the *InterPro* consortium.

The predicted results are exhibited on Table 16.

Table 16- Predicted domains of the proteins. MW represents the molecular weight, in dalton; pI is the isoelectric point; DOM indicates the designation of the different domains and AA No is the number of amino acids associated to those domains

Proteins	MW (Da)	pI		Predicting domains	
Dispersin B	38986.87	5.61	DOM	Glycoside hydrolase family 20, catalytic domain	
			AA No	20-337	
gp49	77279.72	5.11	DOM	Pectin lyase fold/virulence factor	
			AA No	346-475	
gp146	25325.68	8.81	DOM	Peptidoglycan binding-like	Glycoside hydrolase, family 19, catalytic domain
			AA No	3-38	141-200
holin Lambda	11261.13	8.82	DOM	Bacteriophage lambda, GpS, holin	
			AA No	1-101	
holin T1	7577.70	9.30		-	
holin T4	25175.74	7.70	DOM	Bacteriophage T4, GpT, holin	
			AA No	6-217	
holin T7	7391.66	6.08	DOM	Bacteriophage T7, Gp17.5, holin	
			AA No	4-63	
Rz T1	14176.45	8.77		-	
Rz1 Lambda	6588.08	9.06	DOM	Lipoprotein Rz1	
			AA No	20-60	

The analysis of Table 16 allows infer the function of proteins, whose predicted domains are known and conserved. Dispersin B presents hydrolase activity, hydrolyzing O-glycosyl compounds; gp49 cleave pectin using β-elimination mechanism, specific for acidic polysaccharides; gp 146 is a lytic enzyme that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety; holins act against the host cell membrane to allow lytic enzymes of the phage to reach the bacterial cell wall and Rz1 Lambda presents fusogenic properties.

Despite the Alginate Lyase not be present in this table, its function is known. As an endolytic enzyme it is able to cleave alginate polymers strand by the ß-elimination of the 4-O-glycosidic bond to yield two shorter saccharides polymers.

3.1.3 Codon usage

The classification of a codon as rare strongly depends on the expression host. The frequency of the codon usage reflects the abundance of their cognate tRNAs. Rare codons, usually, tend to be in genes expressed at a low level. Thus, when the codon usage of a target protein differs significantly from the average codon usage of the expression host, this could cause problems during expression: decreased mRNA stability; premature termination of transcription and/or translation; frameshifts, deletions and misincorporations and inhibition of protein synthesis and cell growth.

For each protein, the number of each codon type was obtained, using the online tool *Sequence Manipulation Suite: Codon Usage* (http://www.bioinformatics.org/sms2/codon_usage.html), and compared with rare codons that have been associated with translation problems on *E.coli* strains[174].

Table 17 displays the rare codons on E. coli strains presented on each protein.

Table 17- Rare codons on *E. coli* strains present on each protein.

		Rare Codons									
		AGG	AGA	CGG	CGA	GGA	AUA	CUA	CCC	**Total**	**%**
Proteins	dispersin B	0	1	2	3	6	4	1	2	19	5.5
	gp49	10	9	6	2	7	19	0	14	67	9.4
	gp146	1	3	2	0	7	0	1	3	17	7.2
	holin Lambda	0	2	0	0	2	1	1	0	6	5.7
	holin T1	1	1	0	0	1	0	1	0	4	5.6
	holin T4	2	4	1	0	7	11	2	0	27	12.4
	holin T7	0	0	0	1	0	0	1	0	2	3.0
	Rz T1	0	0	0	1	2	0	2	0	5	3.8
	Rz1 Lambda	0	1	0	0	0	1	1	3	6	10.0

3.2 Laboratory Tests

3.2.1 Colony PCR

The results of Colony PCR, done to confirm the transformation on *E. coli*, are showed on Figure 7.

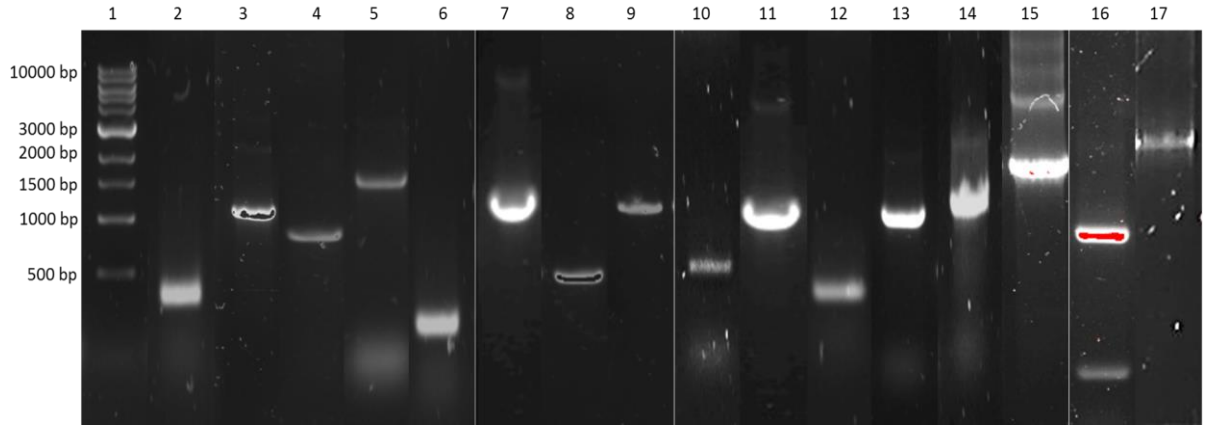


Figure 7- Composion of the 1% agarose gels resulted from Colony PCR from each gene. Column 1 – 1 Kb ladder; column 2 – holin T1; column 3 – holin T1_146; column 4 – holin T4; column 5 – holin T4_146; column 6 – holin T7; column 7 – holin T7_146; column 8 – holin Lambda; column 9 – holin Lamda_146; column 10 – Rz T1; column 11 – Rz T1_146; column 12 - RZ1 Lambda; column 13 – RZ1 Lambda_146; column 14 – dispersin B; column 15 – dispersin B_146; column 16 – gp146; column 17 – gp49.

The gene of holin Lambda_L30_146 was not successfully cloned, probably due to the long size of primers used, resulting in a low percent of correct primers in length and sequence, significantly

decreasing the chances of correct cloning. The other genes were efficaciously cloned, since the corresponding band displays the correct size and this was confirmed by sequencing.

3.2.2 Protein expression and Purification

The aim of recombinant protein expression is the achievement of soluble and active product. To improve the proteins expression conditions, different approaches were tested:

-Application of NaCl 2 M and ethanol. The salting in phenomenon increases the solubility of protein, since the additional ions shield the multiple ionic charges of proteins, weakening the attractive forces between individual protein molecules[175]. Also, the increase in the osmotic pressure, caused by NaCl 2M and ethanol, leads to the accumulation of osmoprotectants, such as glycine betaine, which stabilize the native protein structure. The ethanol induces the expression of heat-shock proteins, preventing protein misfolding and aggregation[176].

- Addition of a compound with azide before the IPTG induction. Sodium azide is a specific inhibitor of the ATPase activity of motor protein SecA, retarding the translocation of fusion protein precursors and increasing the solubility[177].

- Utilization of 1% glucose. When *E. coli* cells grow in a medium containing lactose as the only carbon source, some of the lactose is converted to allolactose, which acts as an inducer of the *lac* operon. The addiction of glucose represses the induction of this operon by lactose, until they consume all the glucose[178].

- Increase/decrease of the O.D. at the induction with IPTG. The T7 promoter often leads to very high production of protein and, in response, this one becomes insoluble. An induction at O.D.600 = 0.400 is a good option to improve the protein expression[179]. However, at this stage, the bacteria are usually in the exponential growth phase, and the chaperones that help on removing of misfolded proteins could not be completely expressed, resulting on a large proportion misfolded protein. The increasing of cell density, to achieve maximum productivity, can frequently cause several major problems, including plasmid loss, significant pH reduction because of cell metabolites, and limited availability of dissolved oxygen[180].

- Increase/decrease of the temperature during the overnight incubation. Temperature decrease leads to a slower expression of the protein, reducing the probability of inclusion bodies formation, consequently increasing solubility[174].

- Increase/decrease the shaking velocity for oxygen variation. Elevated oxygen pressure or rapid increases in oxygen content can cause oxidative stress within the cells, leading to oxidation of specific proteins[181].

The parameters that enable better results for protein expression were: inoculums, with 1% glucose, in BL21 gold (DE3) incubated until $O.D._{600}$ = 0.600. The culture was storage at 4 °C, for 10 min and after induction with IPTG, the incubation occurred at 16 °C and 250 rpm. No others compounds (NaCl 2M, Ethanol and Azide) were used.

Despite the attempts, multiple proteins were not able to be expressed: holin T1, holin T4_146, holin T7, holin T7_146, holin Lambda_L30_146, RzT1_146, Rz1 Lambda and Rz1 Lambda_146.

This may occurred in response of a non-acceptable expression by the metabolic system of the host and consequent cellular stress. The expression of proteins with TMH, due to their hydrophobic residues, is often toxic to the bacterial host, and this may have caused the low expression levels of holin Lambda and spanin Rz T1, or no expression at all in the cases of holins T1, T4_146, T7, T7_146 and spanin RzT1_146[182].

Also the SP influences the protein secretion and an inefficient SP or an incomplete cleavage result on the misfolding of a significant portion of the expressed protein, causing an accumulation of target proteins into insoluble aggregates, known as inclusion bodies or in protein degradation, and, consequently, proteins become biologically inactive. In fact, the presence of a SP (Table 15) might explain why the Rz1 Lambda and Rz1 Lambda_146 were not expressed and present one more reason to the failure on the holin T1 expression[183].

The Table 16 analysis allows inferring that the recombinant protein expression is weak or fails for proteins with MW lower, which is consistent with reported cases in the literature[184].

Disulfide bonds are related to the increase of the proteins stability and the possible erroneous formation may avoid the proteins from attaining their biologically active three-dimensional conformation, once the mispairing of cysteines is an inherent problem, which can cause misfolding, aggregation and low yields during protein production[185].

Also the production of unregulated foreign proteases, responsible for peptide bond cleavage, represents a critical stress and often results in the formation of inclusion bodies, non-expression, or cytotoxicity[186].

The observed low levels of expression or no expression at all and the loss of biological activity may be triggered by the presence of rare codons, associated with translation problems on *E. coli* strains, in the proteins[187].

The remaining proteins were tested on planktonic and sessile cells. The SDS-PAGE performed to the expressed protein is presented on Figure 8.

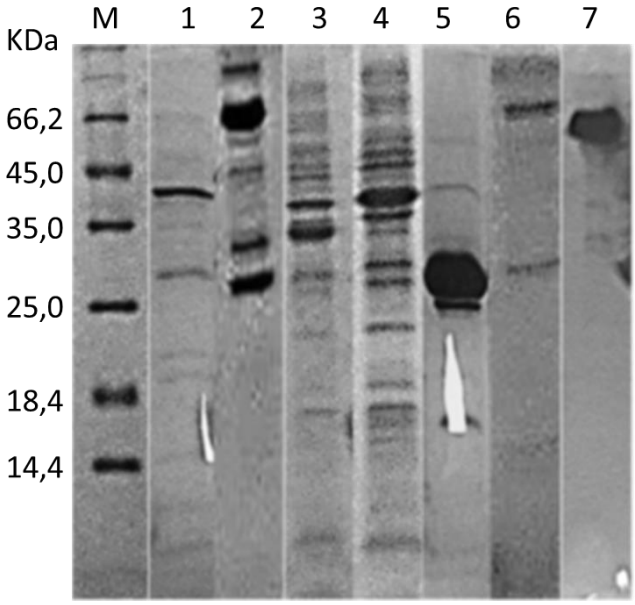


Figure 8- SDS-PAGE of the expressed proteins. M - protein marker, 1- dispersin B (40 kDa); 2- dispersin B_146 (67 kDa); 3- holin T1_146 (36 kDa); 4- holin Lambda_146 (39 kDa); 5- gp146 (28 kDa); 6- holin T4 (26 kDa); and 7- gp49 (78 kDa).

3.2.3 Protein activity tests

The evaluation of the potential of the proteins to combat planktonic and sessile cells was assessed through CFU's count, and respective calculation of CFU/ml, using the below equation, where de Dilution Factor is the inverse of the dilution on which the colonies were counted and the volume of the drop pipetted to LB plate is 0.01 ml.

$$\mathbf{CFU/ml} = \frac{\mathbf{number\ of\ colonies}}{\mathbf{volume\ (mL)}} \times \mathbf{Dilution\ Factor}$$

Planktonic

The amount of CFU/ml of planktonic cells is presented on Table 18 and Figure 9.

Table 18- CFU/ml of planktonic cells, after proteins action.

		Proteins						
		Control	gp146	holin T4	holin Lambda	holin Lambda_146	holin T1_146	Rz1 T1
BACTERIA	ATCC 10145	$2.0*10^4$	$1.0*10^5$	$1.0*10^4$	0	$2.0*10^4$	$2.0*10^4$	0
	PAO1	$5.0*10^4$	$8.0*10^4$	$1.0*10^5$	$5.0*10^4$	$2.0*10^4$	0	$2.0*10^4$
	S1400	0	$1.0*10^5$	$3.6*10^5$	$4.0*10^5$	0	0	0
	BL21	$1.6*10^5$	$3.0*10^4$	$5.0*10^4$	$9.0*10^4$	$8.0*10^4$	$7.0*10^4$	$7.0*10^4$

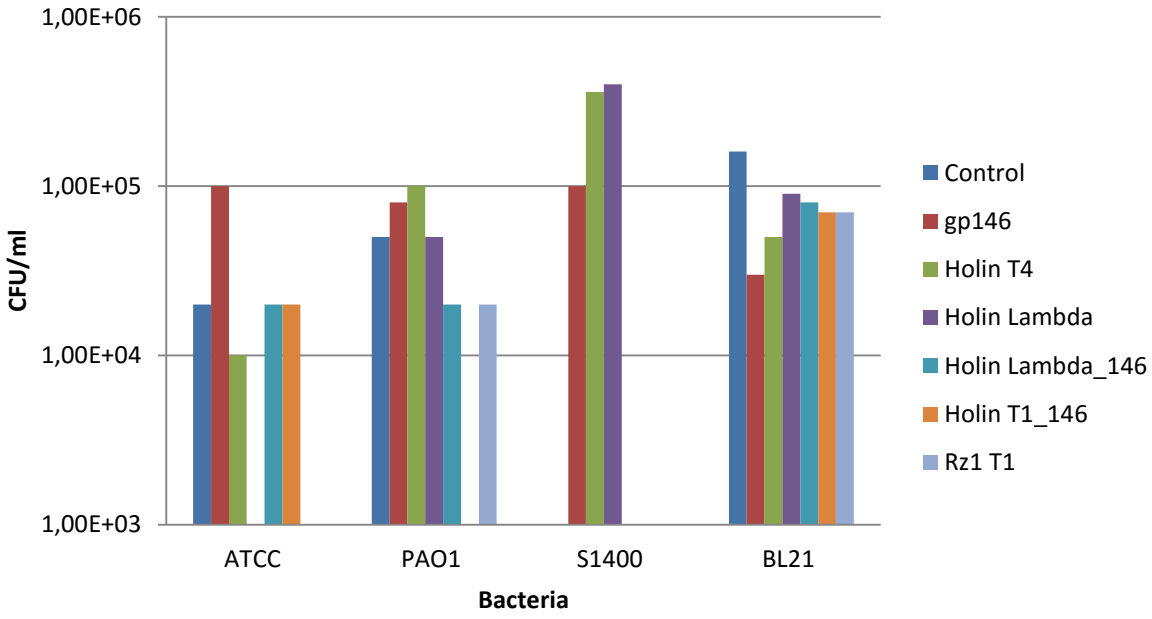


Figure 9- CFU/ml, on a logarithmic scale, from the proteins tests on planktonic cells.

Holin Lambda and Rz T1 showed to be very efficient on reducing the number of cells of ATCC 10145, and holin T4 also promoted reduction of the amount of CFU per ml however, relatively to the control, is not sufficient to this protein be considered as efficient. The growth of PAO1 is completely inhibited by the holin T1_146 and slight reduced by holin Lambda_146. The action of the proteins on S1400 can not be assessed, since the value of CFU per ml on the control is 0. Only a slight decrease was produced by the proteins on cells of *E. coli* BL21. The remaining proteins were ineffective on the combat of the planktonic bacteria.

Despite the dialyzed holin Lambda and spanin Rz1 T1 do not appear on SDS-PAGE, in a first stage, the filtered fraction was loaded on a gel and a band, with the desired size, was visualized. This fact, allied

with the total inhibition of ATCC 10145 cells, might indicate a low expression, to the point of not being visible on gels, but still effective in cell reduction.

The truncated gp146, observed on Figure 8, may be the result of the presence of two start codons in the protein sequence, originating two proteins. The influence on the protein activity was not assessed.

Considering the predicted domains, it was expectable higher cell reductions in the application of those proteins. Many factors may have affected proteins activity. The main causes could be the low amount of soluble protein expressed, problems associated with the folding, the pH of the medium or the presence of foreign proteases. Another reason that may explain the absent of CFU decrease is the cells sensitivity to these proteins. In fact, proteins act differently on each cell and the absent of activity could be the inability to achieve their target.

Beyond the previously mentioned reasons related with the protein expression, the inactivity of gp146 and holins T4, Lambda_146 and T1_146 could be explained by the chosen buffer, due to its pH, and the His-tag, which might have negative effects on the protein folding and its activity. Also the utilization of the *E.coli* strains to protein expression could be one potential cause of the inactivity of proteins, once has limited post-translational modifications and these ones play crucial roles in the assembly, degradation, structure and function of expressed genes[188,189]. Purification conditions or over-purification can lead to removal of specific lipids that may be associated with the protein in the native membrane and essential for its activity[190].

Biofilm

The action of proteins on biofilms can be seen in two perspectives: on the disruption of biofilms (liberating the cells from the biofilm to the medium), on the viability of the biofilm cells (by reducing the number of cells). The first one was assessed by determining the CFUs of the cells in suspension after biofilm incubation with the proteins tested and the second one by determining the CFUs of the cells in biofilm after protein activity, sonication and scraping the wells. Hereupon, the amount of CFU's, relatively to the control, in the first should be higher while in the second one should be lower showing that the proteins were able to detach and remove the cells from the biofilm to suspension reducing the total biofilm. Considering these observations, the best results are obtained when the high values of CFU/ml for the first essay matches with the low quantities on the second.

The Table 19 and Figure 10 illustrate the results obtained to the first approach.

Table 19- CFU/ml of the sessile cells released from the biofilm. The UC (uncountable) represent an amount of CFU higher than 200.

		Results to the tested proteins (CFU/ml)				
		Control	Dispersin B	Dispersin B_146	Alginate Lyase	gp49
Bacteria	ATCC 10145	$6.0*10^{7}$	$1.7*10^{8}$	$2.6*10^{8}$	$1.5*10^{8}$	$2.0*10^{7}$
	PAO1	$3.6*10^{8}$	$3.7*10^{8}$	$4.5*10^{8}$	$6.9*10^{8}$	$7.2*10^{8}$
	S1400	$1.8*10^{8}$	$1.3*10^{8}$	$1.2*10^{8}$	$2.0*10^{8}$	$3.6*10^{8}$
	BL21	$2.7*10^{8}$	$4.0*10^{8}$	$3.3*10^{8}$	#UC	$9.0*10^{7}$

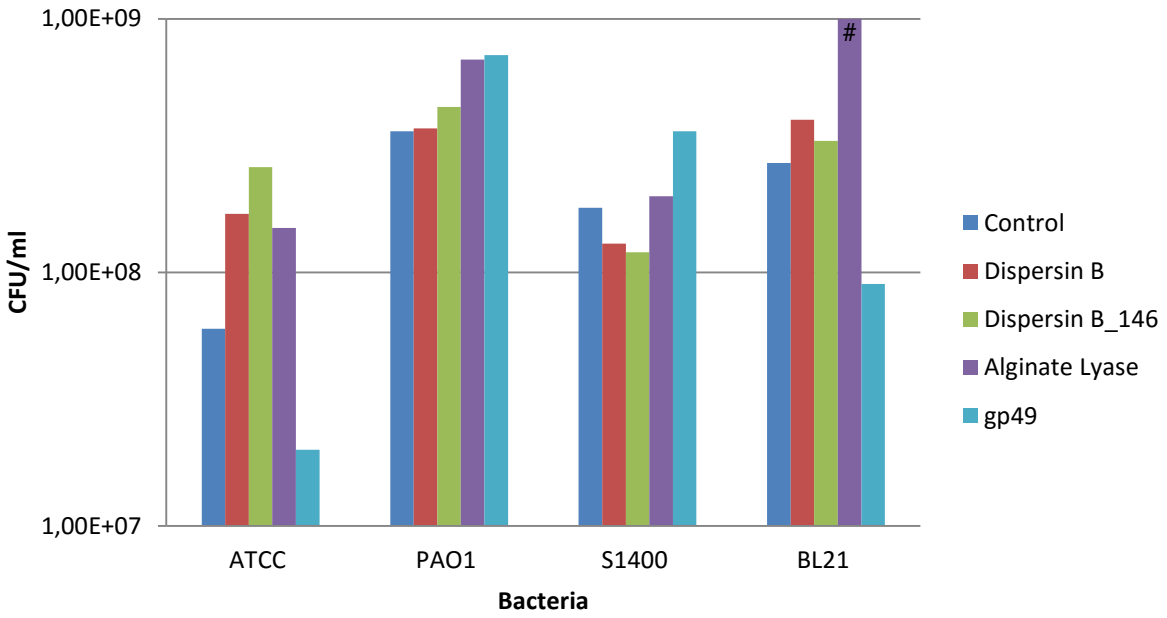


Figure 10- CFU/ml, on a logarithmic scale, of the sessile cells released due to the proteins activity. The '#' represents an amount of CFU higher than 200.

The best result obtained was with the Alginate Lyase on BL21, whose CFU values were higher than 200, resulting in a large number of released cells and, possibly, on a log reduction on the amount of CFU per ml. The other proteins were unable to detach the cells from the biofilm when compared to the control.

The results of the second approach are resumed at Table 20 and Figure 11.

Table 20- CFU/ml of the cells composing the biofilm. The UC represent an amount of CFU higher than 200.

		Results to the tested proteins (CFU/ml)				
		Control	Dispersin B	Dispersin B_146	Alginate Lyase	gp49
Bacteria	ATCC 10145	$1.7*10^8$	$2.9*10^8$	$5.4*10^8$	$1.1*10^8$	$8.0*10^7$
	PAO1	$4.0*10^8$	$4.4*10^8$	$4.4*10^8$	$2.0*10^8$	$4.0*10^8$
	S1400	$1.6*10^8$	$1.0*10^8$	$1.0*10^8$	$1.1*10^8$	$2.7*10^8$
	BL21	#UC	#UC	$5.0*10^8$	$3.8*10^8$	#UC

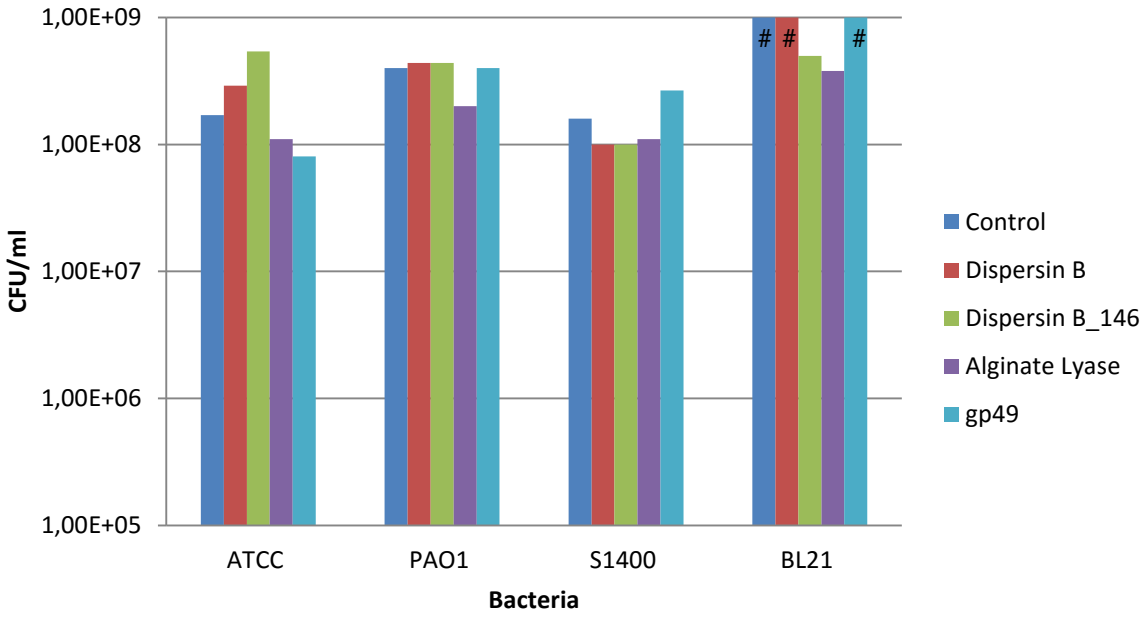


Figure 11- CFU/ml, on a logarithmic scale, of the cells in biofilm, after the proteins activity. The '#' represents an amount of CFU higher than 200.

Despite the evidence that these proteins, at these conditions, are not effective on the combat of biofilms of ATCC 10145, PAO1, S1400 and BL21, since the quantity of CFU per ml, to the different cells was similar to the control, it is also obvious that in ATCC 10145 cells, the protein gp49 produced a slight decrease on the cells in biofilm.

The use of Alginate Lyase proved to be the most efficient protein to disrupt the BL21 biofilm, since it allowed the major release of the cells and at least prevented the biofilm expansion and growth when compared to the control.

Considering the functional domains attributed by the bioinformatics analysis it was expected a higher activity of the proteins in the biofilm cells, as it should happen with the tests with planktonic cell. Once again this may be justified by the points associated with the proteins inactivity already mentioned.

4. Conclusion and Futures Perspectives

Recombinant heterologous expression of the selected proteins proved to be very hard and did not produce the expected and the desired results, despite the multiples approaches (addiction of NaCl 2M, ethanol, azide, glucose; increase/decrease of O.D., temperature and shaking velocity; and tests on different cells host).

To achieve the cloning of holin Lambda L30_146 new primers should be purchased with a higher degree of purity giving its length.

Holins T1, T4_146, T7 and T7_146 and spanins RzT1_146, Rz1 Lambda and Rz1 Lambda_146 were not expressed at sufficient levels to be detected in the SDS-PAGE. To address this issue different expression systems could be used, such as the cell-free, wherein the expression is performed without the use of living cells. This system is suitable for making proteins that are toxic to expression hosts *in vivo*, expression of proteins with modified amino acids and incorporation of post-translational modifications[174].

Relatively to the expressed proteins tested on planktonic cells, since that holin Lambda and Rz T1 inhibited completely the ATCC 10145 cells, and holin T1_146 destroyed the PAO1 cells, it can be concluded that these proteins are active. Therefore, the insensibility of PAO1, S1400 and BL21 to holin Lambda and Rz T1, and of ATCC 10145, S1400 and BL21 to holin T1_146 could be a reason to these proteins not being affected by their action. The remaining three proteins (gp146 and holins T4 and Lambda_146) showed no effect on ATCC 10145, PAO1, S1400 and BL21 cells.

Biofilms of BL21 were efficiently disrupted by the enzyme Alginate Lyase, although that only moderately, confirming the activity of the enzyme. More tests should be carried with higher concentration of protein, different buffer and different incubation times to assess the ability to optimize the protein action.

Several techniques could be implemented to improve the expression and activity of proteins

The temperature reduction, to limit the *in vivo* aggregation of recombinant proteins, is a strategy effective on improving the solubility of difficult proteins and decreasing toxicity to the host organism, benefiting the folding and eliminating, partially, the heat shock proteases that are induced under overexpression conditions. However, an abrupt decrease in cultivation temperature inhibits replication, transcription and translation and affects the promoters' efficiency. A recently principle based on co-expression of the target protein with chaperones from a psychrophilic bacterium (Cpn60 and Cpn10 from *Oleispira antarctica* RB8T) allows protein expression and folding at 4 °C, on *E. coli*[91].

For future projects, the control of pH must be one of the controlled topics, since extreme pH or pH values close to the isoelectric point of the protein affect the stability of the proteins, and, consequently, proteins tend to precipitate. If necessary, a new buffer, with a different pH, must be used[175].

Strong promoters, as the T7-lac promoter used by pET28-a, tends to lead to a "leaky" expression, *i.e.*, there is some level of expression even in the absence of induction. To toxic proteins, this could be problematic, once that a selective pressure to get rid of the protein is create, leading to the accumulation of mutations in the promoter or the cloned gene. In these cases, a modest growth and expression rate is beneficial to avoid the formation of inclusion bodies and can be attained with a weak promoter or by a pLysS or pLysE plasmid, which expresses T7 lysozyme, an inhibitor of T7 RNA polymerase[192].

Since the codon usage is one of the most important factor to protein expression, and it was confirmed the existence of rare codons in the proteins tested (relative to *E. coli*), the change of expression host to *E. coli* Rosetta (DE3) or *E. coli* BL21 (DE3) Codon Plus, which correct for codon bias, it is a hypothesis[182]. As last resource, the expression on yeasts, namely, on *Saccharomyces cerevisiae* and *Pichia pastoris*, could be considered, since this expression system is associated to high yield and high productivity[193].

To control the undesirable proteolysis it may be necessary to add a cocktail of protease inhibitors to the cell suspension.

Also His-tag may affect protein activity, although the relatively small size and charge of the polyhistidine affinity tag ensure that is really rare[194,195]. If confirmed, a technique to solve this problem is the replacing of the His-tag on the other terminus of the protein, or removing it after purification. If the problem persists, another tag or a different purification method should be chosen[185].

In conclusion, this work showed that bacteriophages proteins have high potential to be used in the combat of GN bacterial pathogens, in planktonic cells and also in biofilms. Although, considering the obtained results, their expression can be rather difficult and further work should be carried taking into account the suggested approaches to improve the proteins expression and activity.

BIBLIOGRAPHY

1. Murray PR, Rosenthal KS, Pfaller MA. *Medical Microbiology*. 7th ed. Elsevier Saunders; 2013.

2. Beveridge TJ. Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *J Bacteriol*. 1999;181(16):4725-4733.

3. Beveridge TJ, Schultze-Lam S. The response of selected members of the archaea to the Gram stain. *Microbiology*. 1996;142(10):2887-2895. doi:10.1099/13500872-142-10-2887.

4. Beveridge TJ. Mechanism of gram variability in select bacteria. *J Bacteriol*. 1990;172(3):1609-1620.

5. Kallen AJ, Srinivasan A. Current epidemiology of multidrug- resistant gram- negative bacilli in the United States. *Infect Control Hosp Epidemiol*. 2010;31(S1):51-54. doi:10.1086/655996.

6. Wong PHP, Krosigk M Von, Roscoe DL, Lau TTY, Yousefi M, Bowie WR. Antimicrobial co-resistance patterns of gram-negative bacilli isolated from bloodstream infections : a longitudinal epidemiological study from 2002 – 2011. *BMC Infect Dis*. 2014;14(393):1-10. doi:10.1186/1471-2334-14-393.

7. Bush LM, Perez MT. *Gram-Negative Bacilli- Introduction*.; 2015.

8. Silhavy TJ, Kahne D, Walker S. The Bacterial Cell Envelope. *Cold Spring Harb Perspect Biol*. 2010;2:1-16.

9. Needham BD, Trent MS. Fortifying the barrier : the impact of lipid A remodelling on bacterial pathogenesis. *Nat Rev Microbiol*. 2013;11(7):467-481. doi:10.1038/nrmicro3047.

10. Azzopardi EA, Azzopardi E, Camilleri L, et al. Gram Negative Wound Infection in Hospitalised Adult Burn Patients-Systematic Review and Metanalysis-. *PLoS One*. 2014;9(4):1-7. doi:10.1371/journal.pone.0095042.

11. Kruth SA. Gram-Negative Bacterial Infections. In: *Infectious Diseases of the Dog and Cat*. 3rd ed. ; 2012:321-330.

12. Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System. Overview of Nosocomial Infections Caused by Gram-Negative Bacilli. *Healthc Epidemiol*. 2005;41:848-854.

13. Willcox MDP. Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. *Optom Vis Sci*. 2007;84(4):273-278. doi:10.1097/OPX.0b013e3180439c3e.

14. Tamma PD, Cosgrove SE, Maragakis LL. Combination Therapy for Treatment of Infections with Gram-Negative Bacteria. *Clin Microbiol Rev*. 2012;25(3):450-470. doi:10.1128/CMR.05041-11.

15. Dasgupta N, Arora SK, Ramphal R. fleN , a Gene That Regulates Flagellar Number in Pseudomonas aeruginosa fleN , a Gene That Regulates Flagellar Number in Pseudomonas aeruginosa. *J Bacteriol*. 2000;182(2):357-364. doi:10.1128/JB.182.2.357-364.2000.Updated.

16. Feldman M, Bryan R, Rajan S, et al. Role of Flagella in Pathogenesis of Pseudomonas aeruginosa Pulmonary Infection. *Infect Immun*. 1998;66(1):43-51.

17. Liu P V, Mercer CB. Growth, Toxigenicity and Virulence of Pseudomonas Aeruginosa. *J Hyg (Lond)*. 1963;61:485-491. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2134570&tool=pmcentrez&rendertype=abstract.

18. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-scott HM. Microbial biofilms. *Annu Rev Microbiol*. 1995;49:711-745.

19. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2(2):95-108. doi:10.1038/nrmicro821.

20. Horswill AR, Stoodley P, Stewart PS, Parsek MR. The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. *Anal Bioanal Chem*. 2007;387(2):371-380. doi:10.1007/s00216-006-0720-y.

21. Häusler T. *VIRUSES VS . SUPERBUGS A Solution to the Antibiotics Crisis ?*; 2006.

22. Murray EC, Marek A, Thomson PC, Coia JE. Gram-negative bacteraemia in haemodialysis. *Nephrol Dial Transplant*. 2015;0:1-7. doi:10.1093/ndt/gfv205.

23. Ahmed SS, Oviedo-Orta E, Mekaru SR, Freifeld CC, Tougas G, Brownstein JS. Surveillance for Neisseria meningitidis Disease Activity and Transmission Using Information Technology. *PLoS One*. 2015;10(5):1-17. doi:10.1371/journal.pone.0127406.

24. Ward AC, Bora NM. *Practical Handbook of Microbiology, Second Edition*.; 2008. doi:10.1201/9781420009330.

25. Panizzon JP, Luiz H, Júnior P, et al. Microbial Diversity : Relevance and Relationship Between Environmental Conservation And Human Health. *Brazilian Arch Biol Technol*. 2015;58(1):137-145.

26. Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E. *The Prokaryotes- A Handbook on the Biology of Bacteria*. 3rd ed.; 2006. doi:10.1007/0-387-30745-1.

27. Fitzgerald C. Campylobacter. *Clin Lab Med*. 2015;35(2):289-298. doi:10.1016/j.cll.2015.03.001.

28. Fahey JW, Stephenson KK, Wallace AJ. Dietary amelioration of Helicobacter infection. *Nutr Res*. 2015;35(6):461-473. doi:10.1016/j.nutres.2015.03.001.

29. Collins S, Litt D, Flynn S, Ramsay M, Slack MPE, Ladhani SN. Neonatal invasive Haemophilus influenzae disease in England and Wales: epidemiology, clinical characteristics and outcome. *Clin Infect Dieases*. 2015;60(12):1786-1792.

30. Mercante JW, Winchell JM. Current and Emerging Legionella Diagnostics for Laboratory and Outbreak Investigations. *Clin Microbiol Rev*. 2015;28(1):95-133. doi:10.1128/CMR.00029-14.

31. Harper DR, Parracho HMRT, Walker J, Sharp R, Hughes G, Werthé M. Bacteriophages and Biofilms. *Antibiotics*. 2014;3:270-284. doi:10.3390/antibiotics3030270.

32. Verstraeten N, Braeken K, Debkumari B, et al. Living on a surface: swarming and biofilm formation. *Trends Microbiol*. 2008;16(10):496-506. doi:10.1016/j.tim.2008.07.004.

33. Markowska K, Grudniak AM, Wolska KI. Silver nanoparticles as an alternative strategy against bacterial biofilms. *Acta Biochim Pol*. 2013;60(4):523-530. http://www.ncbi.nlm.nih.gov/pubmed/24432308.

34. Cos, P., Tote, K., Horemans, T., Maes L. Biofilms: an extra hurdle for effective antimicrobial therapy. *Curr Pharm Des*. 2010;16(20):2279-2295.

35. Kostakioti, M., Hadjifrangiskou, M., Hultgren SJ. Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era. *Cold Spring Harb Perspect Med*. 2014;3(4):1-29. doi:10.1101/cshperspect.a010306.Bacterial.

36. Simões M, Carvalho H, Pereira MO, Vieira MJ. Studies on the Behaviour of Pseudomonas fluorescens Biofilms after Ortho-phthalaldehyde Treatment. *Biofouling*. 2003;19(3):151-157. doi:10.1080/0892701031000072127.

37. Simões M, Pereira MO, Vieira MJ. Effect of mechanical stress on biofilms challenged by different chemicals. *Water Res*. 2005;39(20):5142-5152. doi:10.1016/j.watres.2005.09.028.

38. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*. 2001;9(1):34-39. http://www.ncbi.nlm.nih.gov/pubmed/11166241.

39. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001;358(9276):135-138. http://www.ncbi.nlm.nih.gov/pubmed/11463434.

40. Donlan RM, Costerton JW. Biofilms : Survival Mechanisms of Clinically Relevant Microorganisms. *Clin Microbiol Rev*. 2002;15(2):167-193. doi:10.1128/CMR.15.2.167.

41. Anderson, G. G., O'toole GA. Innate and induced resistance mechanisms of bacterial biofilms. *Bact biofilms*. 2008:85-105.

42. Cloete TE. Resistance mechanisms of bacteria to antimicrobial compounds. 1997.

43. Parsek, M. R., Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol*. 2003;57:677.

44. Chen M, Yu Q, Sun H. Novel strategies for the prevention and treatment of biofilm related infections. *Int J Mol Sci*. 2013;14(9):18488-18501. doi:10.3390/ijms140918488.

45. Hancock V, Dahl M, Klemm P. Abolition of biofilm formation in urinary tract Escherichia coli and Klebsiella isolates by metal interference through competition for fur. *Appl Environ Microbiol*. 2010;76(12):3836-3841. doi:10.1128/AEM.00241-10.

46. Davies D. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov*. 2003;2(2):114-122.

47. Toole GO, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol*. 2000;54:49-79.

48. Simoes M. Antimicrobial strategies effective against infectious bacterial biofilms. *Curr Med Chem*. 2011;18(14):2129-2145.

49. Simoes, M., Bennett, R.N., Rosa EA. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Nat Prod Rep*. 2009;26(6):746-757.

50. Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis*. 2002;8(9):881-890. doi:10.3201/eid0809.020063.

51. Beloin C, Roux A, Ghigo J. Escherichia coli biofilms. *Curr Top Microbiol Immunol*. 2008;322:249-289.

52. Karatan E, Watnick P. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev*. 2009;73(2):310-347. doi:10.1128/MMBR.00041-08.

53. Haussler S, Fuqua C. Biofilms 2012: new discoveries and significant wrinkles in a dynamic field. *J Bacteriol*. 2013;195(13):2947-2958. doi:10.1128/JB.00239-13.

54. Bayer a S, Speert DP, Park S, et al. Functional role of mucoid exopolysaccharide (alginate) in antibiotic-induced and polymorphonuclear leukocyte-mediated killing of Pseudomonas aeruginosa. *Infect Immun*. 1991;59(1):302-308. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=257741&tool=pmcentrez&rendertype=abstract.

55. Boyd, A., Chakrabarty AM. Pseudomonas aeruginosa biofilms: role of the alginate exopolysaccharide. *J Ind Microbiol*. 1995;15(3):162-168.

56. Deretic, V., Govan, J. R. W., Konyecsni, W. M., Martin DW. Mucoid Pseudomonas aeruginosa in cystic fibrosis: mutations in the muc loci affect transcription of the algR and algD genes in response to environmental stimuli. *Mol Microbiol*. 1990;4(2):189-196.

57. Gacesa P. Bacterial alginate biosynthesis--recent progress and future prospects. *Microbiology*. 1998;144:1133-1143. http://www.ncbi.nlm.nih.gov/pubmed/9611788.

58. Roychoudhury S, Zielinski NA, Berry A, et al. Alginate synthesis by Pseudomonas aeruginosa : a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin Microbiol Rev*. 1991;4(2):191-206. doi:10.1128/CMR.4.2.191.Updated.

59. Jr WMD. Bacterial adhesion : Seen any good biofilms lately ? *Clin Microbiol Rev*. 2002;15(2):155-166. doi:10.1128/CMR.15.2.155-166.2002.Updated.

60. Banin E, Vasil ML, Greenberg EP. Iron and Pseudomonas aeruginosa biofilm formation. *Proc Natl Acad Sci U S A*. 2005;102(31):11076-11081. doi:10.1073/pnas.0504266102.

61. Anderson GG, Moreau-Marquis S, Stanton B a, O'Toole G a. In vitro analysis of tobramycin-treated Pseudomonas aeruginosa biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun*. 2008;76(4):1423-1433. doi:10.1128/IAI.01373-07.

62. Wu Y, Outten FW. IscR controls iron-dependent biofilm formation in Escherichia coli by regulating type I fimbria expression. *J Bacteriol*. 2009;191(4):1248-1257. doi:10.1128/JB.01086-08.

63. Bhomkar P, Materi W, Semenchenko V, Wishart DS. Transcriptional Response Of E . coli Upon FimH-mediated Fimbrial Adhesion. *Gene Regul Syst Bio*. 2010;4:1-17.

64. Inagaki S, Kuramitsu HK, Sharma A. Contact-dependent regulation of a Tannerella forsythia virulence factor, BspA, in biofilms. *FEMS Microbiol Lett*. 2005;249(2):291-296. doi:10.1016/j.femsle.2005.06.032.

65. Morici L a, Carterson AJ, Wagner VE, et al. Pseudomonas aeruginosa AlgR represses the Rhl quorum-sensing system in a biofilm-specific manner. *J Bacteriol*. 2007;189(21):7752-7764. doi:10.1128/JB.01797-06.

66. Otto K, Silhavy TJ. Surface sensing and adhesion of Escherichia coli controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci U S A*. 2002;99(4):2287-2292. doi:10.1073/pnas.042521699.

67. Sauer K, Cullen MC, Rickard AH, Zeef LAH, Gilbert P, Davies DG. Characterization of Nutrient-Induced Dispersion in Pseudomonas aeruginosa PAO1 Biofilm Characterization of Nutrient-Induced Dispersion in Pseudomonas aeruginosa PAO1 Biofilm. *J Bacteriol*. 2004;186(21):7312-7326. doi:10.1128/JB.186.21.7312.

68. Karatan E, Watnick P. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev*. 2009;73(2):310-347. doi:10.1128/MMBR.00041-08.

69. Amara N, Mashiach R, Amar D, et al. Covalent inhibition of bacterial quorum sensing. *J Am Chem Soc*. 2009;131(30):10610-10619. doi:10.1021/ja903292v.

70. Worthington RJ, Richards JJ, Melander C. Small molecule control of bacterial biofilms. *Org Bomol Chem*. 2012;10(37):7457-7474. doi:10.1039/c2ob25835h.Small.

71. Hentzer M, Wu H, Andersen JB, et al. Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. *EMBO J*. 2003;22(15):3803-3815. doi:10.1093/emboj/cdg366.

72. Adonizio AL, Downum K, Bennett BC, Mathee K. Anti-quorum sensing activity of medicinal plants in southern Florida. *J Ethnopharmacol*. 2006;105(3):427-435. doi:10.1016/j.jep.2005.11.025.

73. Dickschat JS. Quorum sensing and bacterial biofilms. *Nat Prod Rep*. 2010;27(3):343-369.

74. Landini, P., Antoniani, D., Burgess, J. G., Nijland R. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol*. 2010;86(3):813-823.

75. Donlan RM. Biofilms and device-associated infections. *Emerg Infect Dis*. 2001;7(2):277-281. doi:10.3201/eid0702.700277.

76. Stickler DJ. Bacterial Biofilms and the Encrustation of Urethral Catheters. *Biofouling*. 1996;9(4):293-305.

77. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science (80-)*. 1999;284(5418):1318-1322. http://www.ncbi.nlm.nih.gov/pubmed/10334980.

78. Fridkin, S. K., Welbel, S. F., Weinstein RA. Magnitude and prevention of nosocomial infections in the intensive care unit. *Infect Dis Clin North Am*. 1997;11(2):479-496.

79. Archibald, L. K., Gaynes RP. Hospital-acquired infections in the United States: the importance of interhospital comparisons. *Infect Dis Clin North Am*. 1997;11(2):245-255.

80. Huebner, J., Pier, G. B., Maslow, J. N., Muller, E., Shiro, H., Parent, M., Goldmann DA. Endemic Nosocomial Transmission of Staphylococcus epidermidis Bacteremia Isolates in a Neonatal Intensive Care Unit over 1O Years. *J Infect Dis*. 1994;169(3):526-531.

81. Goldmann D a, Huskins WC. Control of nosocomial antimicrobial-resistant bacteria: a strategic priority for hospitals worldwide. *Clin Infect Dis*. 1997;24(1):139-145. http://www.ncbi.nlm.nih.gov/pubmed/8994794.

82. Chattopadhyay MK, Chakraborty R, Grossart H-P, Reddy GS, Jagannadham M V. Antibiotic resistance of bacteria. *Biomed Res Int*. 2015;135(65):1-3. http://epublications.uef.fi/pub/urn_isbn_978-952-61-0097-5/urn_isbn_978-952-61-0097-5.pdf.

83. Arias CA, Murray BE. A New Antibiotic and the Evolution of Resistance. *N Engl J Med*. 2015;372(12):1168-1170.

84. Li X-Z, Plésiat P, Nikaido H. The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. *Clin Microbiol Rev*. 2015;28(2):337-418. doi:10.1128/CMR.00117-14.

85. Lin J, Nishino K, Roberts MC, Tolmasky M, Aminov RI, Zhang L. Mechanisms of antibiotic resistance. *Front Microbiol*. 2015;6(34):1-3. doi:10.1016/S0011-393X(96)80095-6.

86. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J Clin Invest*. 2003;112(9):1300-1307. doi:10.1172/JCI200320074.One.

87. Borges A, Abreu AC, Malheiro J, Saavedra MJ, Simões M. *Biofilm Prevention and Control by Dietary Phytochemicals*.; 2013.

88. Borris RP. Natural products research: perspectives from a major pharmaceutical company. *J Ethnopharmacol*. 1996;51(1-3):29-38.

89. Sakanaka S, Shimura N, Aizawa M, Kim M, Yamamoto T. Preventive Effect of Green Tea Polyphenols against Dental Caries in Conventional Rats. *Biosci Biotechnol Biochem*. 1992;56(4):592-594. doi:10.1271/bbb.56.592.

90. Cimolai N, Cimolai T. The cranberry and the urinary tract. *Eur J Clin Microbiol Infect Dis*. 2007;26(11):767-776. doi:10.1007/s10096-007-0379-0.

91. Chopra I. The increasing use of silver-based products as antimicrobial agents: A useful development or a cause for concern? *J Antimicrob Chemother*. 2007;59(4):587-590. doi:10.1093/jac/dkm006.

92. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. *Biotechnol Adv*. 2009;27(1):76-83. doi:10.1016/j.biotechadv.2008.09.002.

93. Anunthawan T, de la Fuente-Núñez C, Hancock REW, Klaynongsruang S. Cationic amphipathic peptides KT2 and RT2 are taken up into bacterial cells and kill planktonic and biofilm bacteria. *Biochim Biophys Acta - Biomembr*. 2015. doi:10.1016/j.bbamem.2015.02.021.

94. Donlan RM. Biofilm formation: a clinically relevant microbiological process. *Clin Infect Dis*. 2001;33(8):1387-1392. doi:10.1086/322972.

95. Cirioni O, Ghiselli R, Minardi D, et al. RNAIII-inhibiting peptide affects biofilm formation in a rat model of staphylococcal ureteral stent infection. *Antimicrob Agents Chemother*. 2007;51(12):4518-4520. doi:10.1128/AAC.00808-07.

96. Pavithra, D., Doble, M. Biofilm formation, bacterial adhesion and host response on polymeric implants—issues and prevention. *Biomed Mater*. 2008;3(3):034003.

97. Kurek, A., Grudniak, A.M., Dowjat, A.K., Wolska KI. New antibacterial therapeutics and strategies. *Polish J Microbiol*. 2011;60(1):3-12.

98. Ren D, Zuo R, Barrios AFG, et al. Differential Gene Expression for Investigation of Escherichia coli Biofilm Inhibition by Plant Extract Ursolic Acid. *Appl Environ Microbiol*. 2005;71(7):4022-4034. doi:10.1128/AEM.71.7.4022.

99. Packiavathy, I. A. S. V. , Agilandeswari, P., Musthafa, K. S., Karutha Pandian, S., Veera Ravi A. Antibiofilm and quorum sensing inhibitory potential of Cuminum cyminum and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens. *Food Res Int*. 2012;45(1):85-92.

100. Borges, A., Saavedra, M. J., Simões M. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling*. 2012;28(7):755-767.

101. Davies DG, Marques CNH. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *J Bacteriol*. 2009;191(5):1393-1403. doi:10.1128/JB.01214-08.

102. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. *Science*. 2010;328(5978):627-629. doi:10.1126/science.1188628.

103. Hoffmann N, Lee B, Hentzer M, et al. Azithromycin Blocks Quorum Sensing and Alginate Polymer Formation and Increases the Sensitivity to Serum and Stationary-Growth-Phase Killing of Pseudomonas aeruginosa and Attenuates Chronic P. aeruginosa Lung Infection in Cftr / Mice. *Antimicrob Agents Chemother*. 2007;51(10):3677-3687. doi:10.1128/AAC.01011-06.

104. Booy R, Kroll JS. Is Haemophilus influenzae finished? *J Antimicrob Chemother*. 1997;40:149-153.

105. Yamaguchi K, Delden CVAN. Azithromycin Inhibits Quorum Sensing in Pseudomonas aeruginosa. *Antimicrob agents Chemother*. 2001;45(6):1930-1933. doi:10.1128/AAC.45.6.1930.

106. Hanlon GW, Denyer SP, Olliff CJ, Ibrahim LJ. Reduction in Exopolysaccharide Viscosity as an Aid to Bacteriophage Penetration through Pseudomonas aeruginosa Biofilms. *Appl Environ Microbiol*. 2001;67(6):2746-2753. doi:10.1128/AEM.67.6.2746.

107. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents*. 2007;30(2):118-128. doi:10.1016/j.ijantimicag.2007.04.006.

108. Hughes KA, Sutherland IW, Jones M V, Rutherford D. Biofilm susceptibility to bacteriophage attack : the role of phage-borne polysaccharide depolyrmerase. *Microbiology*. 1998;144:3039-3047.

109. Hughes KA, Sutherland IW, Clark J, Jones M V. Bacteriophage and associated polysaccharide depolymerases – novel tools for study of bacterial biofilms. *J Appl Microbiol*. 1998;85:583-590.

110. Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills Bacillus anthracis. *Nature*. 2002;418(AUGUST):440-445.

111. Sutherland IW, Hughes KA, Skillman LC, Tait K. The interaction of phage and biofilms. 2004;232:2-7. doi:10.1016/S0378-1097(04)00041-2.

112. Maniloff J. *Bacteriophages*.; 2006. doi:10.1002/9780470015902.a0000773.pub3.

113. Nilsson AS. Phage therapy—constraints and possibilities. *Ups J Med Sci*. 2014;119(2):1-7. doi:10.3109/03009734.2014.902878.

114. PN SR. Bacteriophage. 2006.

115. Jassim SAA, Limoges RG. Impact of external forces on cyanophage-host interactions in aquatic ecosystems. *World J Microbiol Biotechnol*. 2013;29(10):1751-1762. doi:10.1007/s11274-013-1358-5.

116. Gopalaiah H. Bacteriophage as Antimicrobial Agents: A Milestone. Kailasam S, ed. *J Indian Acad Oral Med Radiol*. 2013;25(1):40-41. doi:10.5005/jp-journals-10011-1337.

117. De Paepe M, Leclerc M, Tinsley CR, Petit M-A. Bacteriophages: an underestimated role in human and animal health? *Front Cell Infect Microbiol*. 2014;4(March):39. doi:10.3389/fcimb.2014.00039.

118. Campbell A(). . I (pp.). SU. Phage evolution and speciation. *The bacteriophages*. 1988:1-14.

119. Verheust C, Pauwels K, Mahillon J, Helinski DR, Herman P. Contained Use of Bacteriophages : Risk Assessment and Biosafety Recommendations. *Appl Biosaf*. 2010;15(1):32-44.

120. Wagner PL, Waldor MK. Bacteriophage Control of Bacterial Virulence. *Infect Immun*. 2002;70(8):3985-3993. doi:10.1128/IAI.70.8.3985.

121. Baxamusa BN. Lytic Cycle.

122. Sulakvelidze A, Alavidze Z. Minireview: Bacteriophage Therapy. *Antimicrob Agents Chemother*. 2001;45(3):649-659. doi:10.1128/AAC.45.3.649.

123. Golkar Z, Bagasra O, Pace DG. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Ctries*. 2014;8(02):129-136. doi:10.3855/jidc.3573.

124. Merabishvili M, Pirnay J-P, Verbeken G, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. *PLoS One*. 2009;4(3):1-10. doi:10.1371/journal.pone.0004944.

125. Wright a, Hawkins CH, Anggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clin Otolaryngol*. 2009;34(4):349-357. doi:10.1111/j.1749-4486.2009.01973.x.

126. McCallin S, Alam Sarker S, Barretto C, et al. Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. *Virology*. 2013;443(2):187-196. doi:10.1016/j.virol.2013.05.022.

127. Sarker SA, McCallin S, Barretto C, et al. Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology*. 2012;434(2):222-232. doi:10.1016/j.virol.2012.09.002.

128. Sulakvelidze A, Alavidze Z, Glenn J, Jr M. Bacteriophage Therapy. *Antimicrob Agents Chemother*. 2001;45(3):649-659. doi:10.1128/AAC.45.3.649.

129. Jassim SAA, Limoges RG. Natural solution to antibiotic resistance: bacteriophages "The Living Drugs." *World J Microbiol Biotechnol*. April 2014:1-18. doi:10.1007/s11274-014-1655-7.

130. Therapy E. Bacteriophage Therapy of Bacterial Infections : an Update of Our Institute's Experience. *Arch Immunol Ther Exp (Warsz)*. 2000;48:547-551.

131. Marinelli LJ, Fitz-gibbon S, Hayes C, et al. Propionibacterium acnes Bacteriophages Display Limited Genetic Diversity and Broad Killing Activity against Bacterial Skin Isolates. *mbio.asm*. 2012;3(5):1-13. doi:10.1128/mBio.00279-12.Editor.

132. Dennehy JJ. What Can Phages Tell Us about Host-Pathogen Coevolution? *Int J Evol Biol*. 2012;2012:1-12. doi:10.1155/2012/396165.

133. Gill, J. J., Hyman P. Phage choice, isolation, and preparation for phage therapy. *Curr Pharm Biotechnol*. 2010;11(1):2-14.

134. Sheng H, Knecht HJ, Kudva IT, Hovde CJ. Application of bacteriophages to control intestinal Escherichia coli O157:H7 levels in ruminants. *Appl Environ Microbiol*. 2006;72(8):5359-5366. doi:10.1128/AEM.00099-06.

135. Clark JR. Bacteriophage therapy : history and future prospects. *Future Virol*. 2015;10(4):449-461.

136. J. Azeredo WS. The use of phages for the removal of incteous biofilms. *Curr Pharm Biotechnol*. 2008;9(4):261-266.

137. Pastagia M, Schuch R, Fischetti VA, Huang DB. Lysins: the arrival of pathogen-directed anti-infectives. *J Med Microbiol*. 2013;62:1506-1516. doi:10.1099/jmm.0.061028-0.

138. Gilmer DB, Schmitz JE, Euler CW, Fischetti VA. Novel bacteriophage lysin with broad lytic activity protects against mixed infection by Streptococcus pyogenes and

methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother*. 2013;57(6):2743-2750. doi:10.1128/AAC.02526-12.

139. Lu TK, Collins JJ. Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc Natl Acad Sci U S A*. 2009;106(12):4629-4634. doi:10.1073/pnas.0800442106.

140. Ackermann HW. Tailed bacteriophages: The order caudovirales. *Adv Virus Res*. 51:135-201. http://cat.inist.fr/?aModele=afficheN&cpsidt=10681901. Accessed May 31, 2014.

141. Goyal, S. M., Gerba, C. P., Bitton G. Phage ecology. *Wiley Ser Ecol Appl Microbiol*. 1987.

142. Hermoso J a, García JL, García P. Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr Opin Microbiol*. 2007;10(5):461-472. doi:10.1016/j.mib.2007.08.002.

143. Loessner MSDMDMJ. Bacteriophage endolysins as novel antimicrobials. *Futur Microbiol*. 2013;7(10):1147-1171. doi:10.2217/fmb.12.97.Bacteriophage.

144. Karthik K, Muneeswaran NS, Manjunathachar HV. Bacteriophages : Effective Alternative to Antibiotics. *Adv Anim Vet Sci*. 2014;2(3):1-7.

145. Fischetti VA. Bacteriophage endolysins: A novel anti-infective to control Gram-positive pathogens. *Int J Med Microbiol*. 2013;300(6):357-362. doi:10.1016/j.ijmm.2010.04.002.Bacteriophage.

146. Briers Y, Lavigne R. Breaking barriers : expansion of the use of endolysins as novel antibacterials against Gram-negative bacteria. *Future Microbiol*. 2015;10(3):377-390.

147. Morita M, Tanji Y, Mizoguchi K, Soejima A, Orito Y, Unno H. Antibacterial activity of Bacillus amyloliquefaciens phage endolysin without holin conjugation. *J Biosci Bioeng*. 2001;91(5):469-473.

148. Lai MJ, Lin NT, Hu A, et al. Antibacterial activity of Acinetobacter baumannii phage ϕAB2 (LysAB2) against both Gram-positive and Gram-negative bacteria. *Appl Microbiol Biotechnol*. 2011;90:529-539. doi:10.1007/s00253-011-3104-y.

149. Kivelä HM, Daugelavicius R, Hankkio RH, et al. Penetration of Membrane-Containing Double-Stranded-DNA Bacteriophage PM2 into Pseudoalteromonas Hosts. *J Bacteriol*. 2004;186(16):5342-5354. doi:10.1128/JB.186.16.5342.

150. Daugelavicius R, Cvirkaite V, Bakiene E, Gabrenaite-verkhovskaya R, Bamford DH. Penetration of Enveloped Double-Stranded RNA Bacteriophages φ 13 and φ 6 into Pseudomonas syringae Cells. *J Virol*. 2005;79(8):5017-5026. doi:10.1128/JVI.79.8.5017.

151. Briers, Y., Lavigne, R., Plessers, P., Hertveldt, K., Hanssens, I., Engelborghs, Y., Volckaert G. Stability analysis of the bacteriophage ϕKMV lysin gp36C and its putative role during infection. *Cell Mol Life Sci C*. 2006;63(16):1899-1905.

152. Briers Y, Miroshnikov K, Chertkov O, et al. The structural peptidoglycan hydrolase gp181 of bacteriophage φKZ. *Biochem Biophys Res Commun*. 2008;374(4):747-751. http://cat.inist.fr/?aModele=afficheN&cpsidt=20614477. Accessed July 3, 2014.

153. Roach DR, Donovan DM. Antimicrobial bacteriophage-derived proteins and therapeutic applications. *Bacteriophage*. 2015;5(3):1-50. doi:10.1080/21597081.2015.1062590.

154. Lorena Rodríguez-Rubio, Beatriz Martínez, David M. Donovan, Ana Rodríguez PG. *Bacteriophage Virion-Associated Peptidoglycan Hydrolases: Potential New Enzybiotics*.; 2013.

155. CALDENTEY J, BAMFORD DH. The lytic enzyme of the Psedomonase phage Ø6. Purification and biochemical characterization. *Biochim Biophys acta Protein Struct Mol Enzymol*. 1992;1159(1):44-50. http://cat.inist.fr/?aModele=afficheN&cpsidt=4313341. Accessed July 3, 2014.

156. Fischetti VA. Bacteriophage Lysins as Effective Antibacterials Vincent. *Curr Opin Microbiol*. 2009;11(5):393-400. doi:10.1016/j.mib.2008.09.012.Bacteriophage.

157. Wang IN, Smith DL, Young R. Holins: the protein clocks of bacteriophage infections. *Annu Rev Microbiol*. 2000;54:799-825. doi:10.1146/annurev.micro.54.1.799.

158. Xu M, Struck DK, Deaton J, Wang I-N, Young R. A signal-arrest-release sequence mediates export and control of the phage P1 endolysin. *Proc Natl Acad Sci U S A*. 2004;101(17):6415-6420. doi:10.1073/pnas.0400957101.

159. Young R. Bacteriophage lysis: mechanism and regulation. *Microbiol Rev*. 1992;56(3):430-481. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=372879&tool=pmcentrez&rendertype=abstract.

160. Park T, Struck DK, Deaton JF, Young R. Topological dynamics of holins in programmed bacterial lysis. *Proc Natl Acad Sci U S A*. 2006;103(52):19713-19718. doi:10.1073/pnas.0600943103.

161. Rice KC, Bayles KW. Molecular control of bacterial death and lysis. *Microbiol Mol Biol Rev*. 2008;72(1):85-109. doi:10.1128/MMBR.00030-07.

162. Maciejewska B. Bacteriophages and Phage-Derived Proteins – Application Approaches. *Curr Med Chem*. 2015;22:1757-1773.

163. Young R. Phage lysis: three steps, three choices, one outcome. *J Microbiol*. 2014;52(3):243-258. doi:10.1007/s12275-014-4087-z.

164. Tait K, Skillman LC, Sutherland IW. The efficacy of bacteriophage as a method of biofilm eradication. *Biofouling*. 2002;18(4):305-311. doi:10.1080/0892701021000034418.

165. Sillankorva S, Pleteneva E, Shaburova O, et al. Salmonella Enteritidis bacteriophage candidates for phage therapy of poultry. *J Appl Microbiol*. 2010;108(4):1175-1186. doi:10.1111/j.1365-2672.2009.04549.x.

166. Pires D, Sillankorva S, Faustino A, Azeredo J. Use of newly isolated phages for control of Pseudomonas aeruginosa PAO1 and ATCC 10145 biofilms. *Res Microbiol*. 2011;162(8):798-806. doi:10.1016/j.resmic.2011.06.010.

167. White R, Chiba S, Pang T, et al. Holin triggering in real time. *Proc Natl Acad Sci U S A*. 2011;108(2):798-803. doi:10.1073/pnas.1011921108.

168. BioRad. Gel Doc ™ XR + and ChemiDoc ™ XRS + Systems with Image Lab ™. 2014:236.

169. Armen R, Alonso DOV, Daggett V. The role of α-, 310-, and π-helix in helix→coil transitions. *Protein Sci*. 2003;12(6):1145-1157. doi:10.1110/ps.0240103.peptides.

170. Chen CP, Kernytsky A, Rost B. Transmembrane helix predictions revisited. *Protein Sci*. 2002;11(12):2774-2791. doi:10.1110/ps.0214502.al.

171. Zhang Z, Henzel WJ. Signal peptide prediction based on analysis of experimentally verified cleavage sites. *Protein Sci*. 2004;13(10):2819-2824. doi:10.1110/ps.04682504.

172. Viklund H, Elofsson A. Best alpha-helical transmembrane protein topology predictions are achieved using hidden Markov models and evolutionary information. *Protein Sci*. 2004;13(7):1908-1917. doi:10.1110/ps.04625404.

173. Yuan Z, Davis MJ, Zhang F, Teasdale RD. Computational differentiation of N-terminal signal peptides and transmembrane helices. *Biochem Biophys Res Commun*. 2003;312(4):1278-1283. doi:10.1016/j.bbrc.2003.11.069.

174. European Molecular Biology Laboratory. Protein expression and purification core facility. https://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/improving_protein_solubility/. Published 2010. Accessed October 28, 2015.

175. Zidani S, Fahloul D, Bacha A. Effects of pH, NaCl, ethanol, and drying methods on the solubility of Saccharomyces cerevisiaeproteins. *CyTA - J Food*. 2012;10(1):42-47. doi:10.1080/19476337.2010.543472.

176. Georgiou G, Valaxt P. Expression of correctly folded proteins in Escherichia coil. *Biochem Eng*. 1996;7(2):190-197.

177. Chen N, Hong F-L, Wang H-H, et al. Modified Recombinant Proteins Can Be Exported via the Sec Pathway in Escherichia coli. *PLoS One*. 2012;7(8):1-8. doi:10.1371/journal.pone.0042519.

178. Stacey G. Microbiology 411: Bacterial Genetics. GENE REGULATION: LAC OPERON.

179. New England BioLabs Inc. Protein Expression with T7 Strains. Protocols.

180. Shiloach J, Fass R. Growing E. coli to high cell density—A historical perspective on method development. *Biotechnol Adv*. 2005;23(5):345-357.

181. Konz JO, King J, Cooney CL. Effects of oxygen on recombinant protein expression. *Biotechnol Prog*. 1998;14(3):393-409.

182. Duong-Ly KC, Gabelli SB. *Explanatory Chapter: Troubleshooting Recombinant Protein Expression:General*. Vol 541. 1st ed. Elsevier Inc.; 2014. doi:10.1016/B978-0-12-420119-4.00017-3.

183. Palomares L a, Estrada-Mondaca S, Ramírez OT. Production of recombinant proteins: challenges and solutions. In: *Methods in Molecular Biology*. Vol 267. ; 2004:15-52. doi:10.1385/1-59259-774-2:015.

184. Mukherjee S, Daifalla N, Liu C, Campos-Neto A. Alternative approach to express Mycobacterium tuberculosis proteins in Escherichia coli. *Biotechniques*. 2003;35(1):34, 36, 38, 40. http://www.ncbi.nlm.nih.gov/pubmed/12866402.

185. Rosano GL, Ceccarelli E a. Recombinant protein expression in Escherichia coli: Advances and challenges. *Front Microbiol*. 2014;5(April):1-17. doi:10.3389/fmicb.2014.00172.

186. Kwon K, Hasseman J, Latham S, et al. Recombinant expression and functional analysis of proteases from Streptococcus pneumoniae, Bacillus anthracis, and Yersinia pestis. *BMC Biochem*. 2011;12(1):17. doi:10.1186/1471-2091-12-17.

187. Cunningham F, Deber CM. Optimizing synthesis and expression of transmembrane peptides and proteins. *Methods*. 2007;41(4):370-380. doi:10.1016/j.ymeth.2006.07.003.

188. Balonova L, Hernychova L. Analysis of Francisella tularensis Glycoproteins. In: *BSL3 and BSL4 Agents: Proteomics, Glycomics, and Antigenicity*. ; 2011:67-78.

189. Broadway N. Recombinant Protein Expression: Vector-Host Systems. 123. http://www.labome.com/method/Recombinant-Protein-Expression-Vector-Host-Systems.html. Published 2012. Accessed October 29, 2015.

190. GE Healthcare. *Purifying Challenging Proteins*. Vol 28-9095-31.; 2007. doi:10.1002/anie.200802483.

191. Gopal GJ, Kumar A. Strategies for the Production of Recombinant Protein in Escherichia coli. *Protein J*. 2013;32(6):419-425. doi:10.1007/s10930-013-9502-5.

192. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol*. 2003;60(5):523-533. doi:10.1007/s00253-002-1158-6.

193. Lee BH. Food safety issues of new biotechnologies. In: *Fundamentals of Food Biotechnology*. ; 2014:467-482.

194. Bornhorst J a., Falke JJ. Purification of Proteins Using Polyhistidine Affinity Tags. *Methods Enzymol*. 2000;326(16):245-254. doi:10.1016/j.pep.2011.08.022.

195. Carson M, Johnson DH, McDonald H, Brouillette C, DeLucas LJ. His-tag impact on structure. *Acta Crystallogr Sect D Biol Crystallogr*. 2007;63(3):295-301. doi:10.1107/S0907444906052024.

ANNEXES

Annex I – Expression vector pET-28a

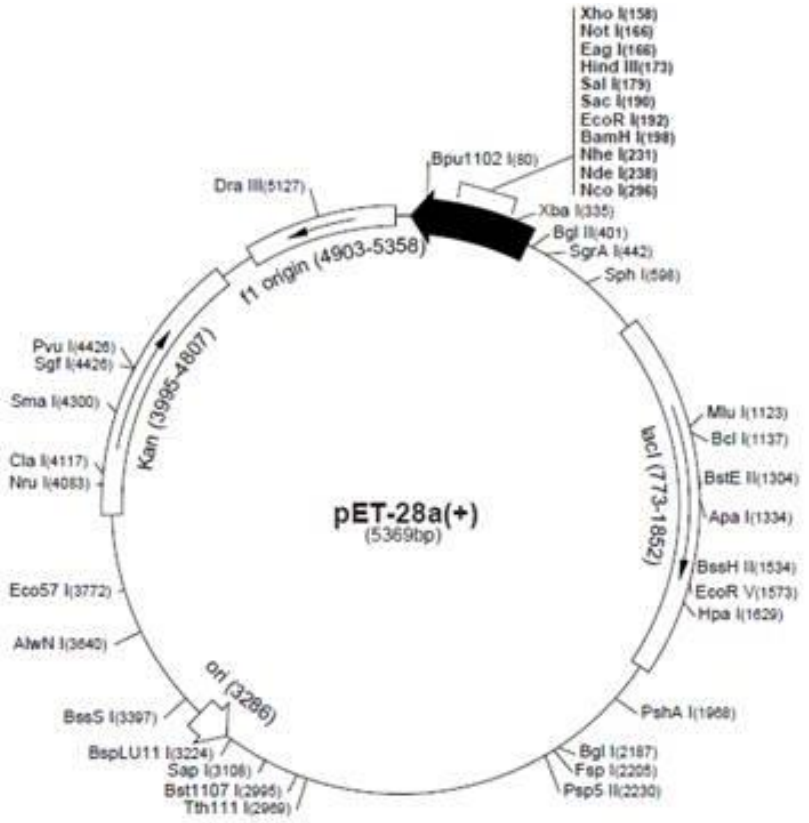


Figure 12- Restriction map of cloning vector used, obtained from http://biochem.web.utah.edu/hill/links/pET28.pdf.

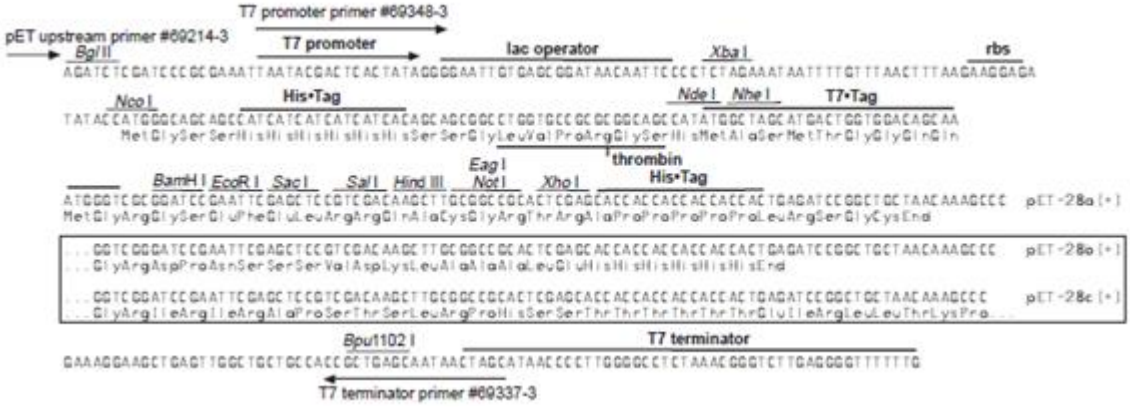


Figure 13- Multiple Cloning site of expression vector pET-28a, obtained from http://biochem.web.utah.edu/hill/links/pET28.pdf.

Annex II – Proteins' Amino Acid sequences

Table 21- Amino Acid sequence of each protein

Proteins	Amino Acid Sequences
holin T1	MKEFLTAATSSTGGASLVGAATGQLYIAGATFICFLLFGAWGAYWKYRDSKAIQEALNDGDLNKALKIRGR
holin T4	MAAPRISFSPSDILFGVLDRLFKDNATGKVLASRVAVVILLFIMAIVWYRGDSFFEYYKQSKYETYSEIIEKERTARF ESVALEQLQIVHISSEADFSAVYSFRPKNLNYFVDIIAYEGKLPSTISEKSLGGYPVDKTMDEYTVHLNGRHYYSN SKFAFLPTKKPTPEINYMYSCPYFNLDNIYAGTITMYWYRNDHISNDRLESICAQAARILGRAK
holin T7	MLSLDFNNELIKAAPIVGTGVADVSARLFFGLSLNEWFYVAAIAYTVVQIGAKVVDKMIDWKKANKE
holin Lambda	MPEKHDLLAAILAAKEQGIGAILAFAMAYLRGRYNGGAFTKTVIDATMCAIIAWFIRDLLDFAGLSSNLAYITSVFIG YIGTDSIGSLIKRFAAKKAGVEDGRNQ
RzT1	MKLKKTCIAITVAVGVISLSGCSTASALSGLLSDSPDVTAQVGAENTKQLAGVTAKADDKREVKVSDSNIGKIDSS VKKSVEVSTIQANTVNAESITVTKSGSWYDPVVCWILVFIVLLLFYFLIRKHEKKEA
Rz1 Lambda	MLKLKMMLCVMMLPLVVVGCTSKQSVSQCVKPPPPPAWIMQPPPDWQTPLNGIISPSERG
gp146	MNAAIAEIQRMLIEGGFSVGKSGADGLYGPATKAALQKCIAQATSGNNKGGTLKLTQAQLDKIFPVGASSGRNA KFLKPLNDLFEKTEINTVNRVAGFLSQIGVESAEFRYVRELGNDAYFDKYDTGPIAERLGNTPQKDGDGAKYKG RGLIQVTGLANYKACGKALGLDLVNHPELLEQPEYAVASAGWYWDTRNINAACDADDIVKITKLVNGGTNHLAE RTAYYKKAKSVLTS
gp49	MADLLPTVKVSDLPTATESFEGDYLVVDQSDATRKSTWSDMFSRFGLMRLFSFQEGGTLVSPKDQVIDRSTNRI YQWTGAYPKLVPADSTPETTGGVGEGAWSANDPSLRGDLAGANGSTFIGGPAGTVAQSLDGFVTPAQFMGKYP TTTEAVTALAAYAKENKKAVLAWGWNLVLETSVYIDGVEWYGGSFNQTGGNRYMYLSNSTFRWVTFTGVCTRH YGGRLIITDSSWVNNTNTAAMLLQALPIEGTIDILDSDFRGCKYGILQQGTGALVTRARFARLNFNDLTGDAIECN VVQRHYKAGGLTIEDINIDNINNTDNSPNWGIGIGVAGQGPYGANASDDQYVSGIIIRNVKMRRVRQCIHFELCR DFKVENVEVYPDASVSNGTLLASGGVVCYGCKDYIIDGVRGEMVNGATRFIYFGWGVNQGTFAAPCRDFTLRNV RTHTGLVDIPVSAMDDWTNDVKVEDIECHTFKYRGLVSKLRLADIRCKQFDGIGDYEAGQGEAGGAMKRWAW CSAEIININSLDDNGVANGKFGQVGFDHLTTYGCNFDVVQHSKTNGNRGVILLNAGNIYISDNDDFPQGKEFVK GDIILKKTGGMFVVETGGSYIEPNDFIKATVVGSKTIECAADSSIRQPWATRAFKSAGLQLTIPGAGPGGADLQTT VIRAPYQKGAWITPFYLDIADPIQTATPDNTALVSTNPVVYSERT
dispersin B	MNCCVKGNSIYPQKTSTKQTGLMLDIARHFYSPEVIKSFIDTISLSGGNFLHLHFSDHENYAIESHLLNQRAENA VQGKDGIYINPYTGKPFLSYRQLDDIKAYAKAKGIELIPELDSPNHMTAIFKLVQKDRGVKYLQGLKSRQVDDEID ITNADSITFMQSLMSEVIDIFGDTSQHFHIGGDEFGYSVESNHEFITYANKLSYFLEKKGLKTRMWNDGLIKNTF EQINPNIEITYWSYDGDTQDKNEAAERRDMRVSLPELLAKGFTVLNYNSYYLYIVPKASPTFSQDAAFAAKDVIK NWDLGVWDGRNTKNRVQNTHEIAGAALSIWGEDAKALKDETI

ANNEX III – GENES' DNA SEQUENCES

Table 22- DNA sequence of each gene

Proteins	DNA Sequences
holin T1	ATGAAAGAGTTTTTAACGGCTGCTACGTCAAGCACTGGCGGTGCTTCGTTGGTAGGGGCGGCGACAGGGCA ACTTTATATTGCTGGCGCTACATTCATTTGCTTTCTGCTTTTTGGTGCCTGGGGAGCGTACTGGAAGTATCGT GATAGCAAGGCAATTCAGGAAGCGTTAAACGATGGCGATCTAAATAAGGCGCTTAAGATCAGGGGGAGATAA
holin T4	ATGGCAGCACCTAGAATATCATTTTCGCCCTCTGATATTCTATTTGGTGTTCTAGATCGCTTGTTCAAAGATAA CGCTACCGGGAAGGTTCTTGCTTCCCGGGTAGCTGTCGTAATTCTTTTGTTTATAATGGCGATTGTTTGGTAT AGGGGAGATAGTTTCTTTGAGTACTATAAGCAATCAAAGTATGAAACATACAGTGAAATTATTGAAAAGGAAAG AACTGCACGCTTTGAATCTGTCGCCCTGGAACAACTCCAGATAGTTCATATATCATCTGAGGCAGACTTTAGT GCGGTGTATTCTTTCCGCCCTAAAAACTTAAACTATTTTGTTGATATTATAGCATACGAAGGAAAATTACCTTC AACAATAAGTGAAAAATCACTTGGAGGATATCCTGTTGATAAAACTATGGATGAATATACAGTTCATTTAAATG GACGTCATTATTATTCCAACTCAAAATTTGCTTTTTTACCAACTAAAAAGCCTACTCCCGAAATAAACTACATG TACAGTTGTCCATATTTTAATTTGGATAATATCTATGCTGGAACGATAACCATGTACTGGTATAGAAATGATCA TATAAGTAATGACCGCCTTGAATCAATATGTGCTCAGGCGGCCAGAATATTAGGAAGGGCTAAATAA
holin T7	GTGCTATCATTAGACTTTAACAACGAATTGATTAAGGCTGCTCCAATTGTTGGGACGGGTGTAGCAGATGTTA GTGCTCGACTGTTCTTTGGGTTAAGCCTTAACGAATGGTTCTACGTTGCTGCTATCGCCTACACAGTGGTTCA GATTGGTGCCAAGGTAGTCGATAAGATGATTGACTGGAAGAAAGCCAATAAGGAGTGA
holin Lambda	ATGCCAGAAAAACATGACCTGTTGGCCGCCATTCTCGCGGCAAAGGAACAAGGCATCGGGGCAATCCTTGC GTTTGCAATGGCGTACCTTCGCGGCAGATATAATGGCGGTGCGTTTACAAAAACAGTAATCGACGCAACGAT GTGCGCCATTATCGCCTGGTTCATTCGTGACCTTCTCGACTTCGCCGGACTAAGTAGCAATCTCGCTTATAT AACGAGCGTGTTTATCGGCTACATCGGTACTGACTCGATTGGTTCGCTTATCAAACGCTTCGCTGCTAAAAAA GCCGGAGTAGAAGATGGTAGAAATCAATAA
RzT1	ATGAAACTTAAGAAAACGTGCATTGCAATTACGGTTGCTGTTGGTGTGATTTCTCTATCCGGTTGTTCGACGG CATCTGCTCTGAGTGGTTTACTTTCTGACTCCCCGGATGTTACGGCGCAGGTTGGCGCTGAGAACACAAAAC AACTAGCAGGAGTAACAGCAAAGGCGGATGATAAGCGAGAAGTGAAGGTGAGTGATTCAAATATTGGCAAGA TTGACTCATCCGTCAAGAAGTCCGTGGAGGTGTCAACCATTCAGGCCAACACGGTTAACGCTGAAAGCATCA CAGTAACCAAATCTGGAAGCTGGTACGATCCTGTGGTTTGCTGGATTCTCGTTTTTATTGTCCTGTTGCTGTT TTATTTTTTAATTCGTAAGCACGAAAAAAAGGAGGCGTAA
Rz1 Lambda	ATGCTAAAGCTGAAAATGATGCTCTGCGTGATGATGTTGCCGCTGGTCGTCGTCGGTTGCACATCAAAGCAG TCTGTCAGTCAGTGCGTGAAGCCACCACCGCCTCCGGCGTGGATAATGCAGCCTCCCCCCGACTGGCAGA CACCGCTGAACGGGATTATTTCACCCTCAGAGAGAGGCTGA
gp146	ATGAATGCTGCAATTGCGGAGATTCAGCGTATGCTGATCGAAGGTGGGTTTAGCGTCGGCAAGTCTGGTGCT GATGGATTGTACGGACCCGCTACAAAAGCCGCACTGCAAAAGTGCATTGCACAGGCTACCAGTGGAAACAAT AAAGGAGGTACTTTGAAACTCACCCAAGCACAACTGGACAAAATCTTCCCCGTTGGTGCAAGTTCTGGGAGG AATGCAAAATTCCTGAAGCCGCTCAATGACCTGTTTGAAAAGACAGAGATTAATACGGTAAATCGGGTTGCAG GATTCCTGTCTCAGATTGGTGTGGAGTCGGCGGAGTTCCGGTATGTACGTGAACTCGGTAACGATGCCTACT TTGACAAGTACGACACTGGTCCTATTGCAGAAAGACTTGGAAACACACCCCAGAAAGATGGGGATGGTGCCA AGTACAAGGGGAGAGGTCTGATTCAGGTGACCGGACTCGCAAACTACAAGGCTTGCGGTAAAGCACTCGGT CTTGACCTCGTTAACCACCCTGAGTTGCTTGAACAGCCTGAGTATGCAGTTGCCAGCGCTGGTTGGTATTGG GACACGAGAAACATCAACGCCGCTTGCGATGCTGATGATATCGTGAAAATTACCAAGCTGGTAAACGGTGGT ACAAATCACCTTGCCGAGCGCACAGCCTATTACAAAAAGGCAAAATCTGTTCTAACCTCGTAA
gp49	TTAAGTCCTTTCGCTGTATACTACGGGATTTGTCGAAACAAGAGCCGTGTTATCAGGTGTGGCTGTCTGGATT GGATCTGCAATGTCCAGATAGAACGGTGTGATCCAAGCACCTTTTTGATATGGCGCTCTTATAACCGTTGTCT GGAGATCCGCACCACCGGGGCCTGCGCCTGGGATGGTGAGTTGCAACCCAGCAGATTTAAATGCACGAGTA GCCCAAGGTTGTCGGATACTTGAATCTGCTGCACACTCAATCGTTTTTGAGCCAACCACAGTTGCCTTGATG AAATCATTAGGTTCAATGTAAGAACCCCCTGTCTCAACAACAAACATACCACCAGTCTTCTTCAGAATGATGT CCCCTTTAACGAATTCCTTTCCTTGAGGAAAATCATCATTATCAGAGATATAGATGTTACCAGCATTAAGCAGT ATGACACCTCTGTTTCCGTTTGTCTTTGAGTGCTGAACCACATCGAAGTTACAACCGTATGTTGTTAAGTGGT CAAAGCCTACCTGACCGAACTTACCGTTAGCGACACCATTATCATCAAGGCTATTGATATTAATGATCTCAGC AGAACACCAAGCCCAACGCTTCATCGCCCCGCCTGCCTCACCCTGTCCAGCCTCATAGTCGCCAATACCAT CAAACTGTTTACAGCGGATGTCAGCAAGACGAAGTTTTGAGACAAGACCGCGATATTTGAAAGTGTGGCACT CAATGTCCTCAACCTTCACATCGTTGGTCCAGTCATCCATAGCACTAACTGGAATATCTACCAGTCCCGTAT GAGTACGTACATTTCGCAGTGTAAAGTCACGGCACGGAGCAGCAAAGGTTCCTTGGTTAACACCCCACCCAA

	AATAAATGAATCGGGTAGCACCGTTGACCATTTCGCCACGAACACCATCAATGATATAGTCTTTGCAACCATA ACATACGACACCACCGGATGCCAGCAGTGTTCCGTTAGAAACGGATGCATCAGGATAAACCTCAACGTTCTC GACCTTAAAGTCACGGCAAAGCTCGAAGTGGATACATTGACGAACTCGACGCATCTTAACGTTACGAATGAT GATCCCACTAACATATTGGTCATCAGAGGCGTTAGCACCATACGGTCCCTGCCCCGCAACACCGATACCAAT ACCCCAGTTAGGAGAGTTATCTGTATTGTTGATGTTGTCGATGTTAATATCTTCGATTGTCAAACCACCCGCT TTATAGTGTCTCTGTACAACGTTACACTAATAGCATCGCCTGTCAAATCGTTAAAGTTGAGACGGGCAAAACG TGCGCGAGTAACAAGAGCGCCTGTCCTTGCTGAAGGATACCATATTTACAACCTCTGAAATCACTATCCAGA ATGTCAATTGTACCTTCTATAGGGAGGGCTTGTAGTAACATCGCTGCGGTGTTGGTATTGTTAACCCATGAGG AGTCTGTAATGATCAGACGCCCACCATAATGGCGAGTACAAACACCTGTGAAGGTCACCCAGCGGAATGTGC TGTTTGATAAATACATGTATCTGTTGCCGCCAGTCTGGTTAAAGCTTCCACCATACCATTCAACACCGTCGAT ATCACACTTGTCTCAAGAACAAGGTTCCATCCCCATGCAAGAACAGCTTTTTTGTTCTCTTTTGCGTATGCAG CTAACGCTGTTACAGCCTCTGTCGTTGTTGGATATTTCCCCATGAATTGTGCAGGGGTAACAAAACCATCGA GTGATTGAGCCACTGTTCCCGCTGGTCCACCGATAAAGGTTGAGCCATTAGCACCTGCAAGGTCTCCACGTA GAGATGGGTCGTTAGCTGACCACGCCCCTTCACCAACACCACCTGTAGTCTCCGGTGTGGAGTCGGCAGGA ACAAGTTTCGGGTATGCCCCTGTCCATTGATAGATCCTGTTGGTTGATCTGTCGATCACCTGATCTTTGGGG GAGACAAGCGTCCCCCCTTCTTGGAAGGAAAACAGGCGCATCAACCCGAAACGGGAGAACATATCTGACCA TGTTGATTTACGGGTTGCATCCGACTGGTCAACAACCAGGTAGTCTCCCTCGAAAGATTCCGTCGCAGTCGG TAAGTCTGAGACCTTGACGGTAGGTAATAGATCTGCCAT
dispersin B	ATGAACTGCTGCGTGAAGGGCAACAGCATCTACCCGCAGAAGACCAGCACCAAGCAGACCGGCCTGATGCT GGACATCGCCCGCCACTTCTACAGCCCGGAGGTGATCAAGAGCTTCATCGACACCATCAGCCTGAGCGGCG GCAACTTCCTGCACCTGCACTTCAGCGACCACGAGAACTACGCCATCGAGAGCCACCTGCTGAACCAGCGC GCCGAGAACGCCGTGCAGGGCAAGGACGGCATCTACATCAACCCGTACACCGGCAAGCCGTTCCTGAGCT ACCGCCAGCTGGACGACATCAAGGCCTACGCCAAGGCCAAGGGCATCGAGCTGATCCCGGAGCTGGACAG CCCGAACCACATGACCGCCATCTTCAAGCTGGTGCAGAAGGACCGCGGCGTGAAGTACCTGCAGGGCCTG AAGAGCCGCCAGGTGGACGACGAGATCGACATCACCAACGCCGACAGCATCACCTTCATGCAGAGCCTGAT GAGCGAGGTGATCGACATCTTCGGCGACACCAGCCAGCACTTCCACATCGGCGGCGACGAGTTCGGCTACA GCGTGGAGAGCAACCACGAGTTCATCACCTACGCCAACAAGCTGAGCTACTTCCTGGAGAAGAAGGGCCTG AAGACCCGCATGTGGAACGACGGCCTGATCAAGAACACCTTCGAGCAGATCAACCCGAACATCGAGATCAC CTACTGGAGCTACGACGGCGACACCCAGGACAAGAACGAGGCCGCCGAGCGCCGCGACATGCGCGTGAGC CTGCCGGAGCTGCTGGCCAAGGGCTTCACCGTGCTGAACTACAACAGCTACTACCTGTACATCGTGCCGAA GGCCAGCCCGACCTTCAGCCAGGACGCCGCCTTCGCCGCCAAGGACGTGATCAAGAACTGGGACCTGGGC GTGTGGGACGGCCGCAACACCAAGAACCGCGTGCAGAACACCCACGAGATCGCCGGCGCCGCCCTGAGCA TCTGGGGCGAGGACGCCAAGGCCCTGAAGGACGAGACCATC

Annex IV – Competent culture $CaCl_2$ method

1. Grow the cells in 10 ml of LB overnight, at 37 °C and 120 rpm.
2. Dilute the suspension 1:100 in fresh LB, to a final volume of 100 ml.
3. Grow the dilution at 37 °C and 200 rpm, until A_{600} = 0.300.
4. Centrifuge the culture for 10 min at 4 °C and 3300 g.
5. Decant supernatant.
6. Resuspend pellet in 50 mL of ice-cold (0°C) 0.1 M $MgCl_2$ (*VWR CHEMICAL, BDH, Prolabo®*).
7. Store the pellet on ice for 30 min.
8. Centrifuge the resuspended pellet for 10 min, at 4°C and 3300 g.
9. Resuspend the pellet with the use of a micro-pipette in 10 mL of ice-cold 0.1M $CaCl_2$ (*AppliChem, BioChemical*).
10. Centrifuge for 10 min at 4 °C and 3300 g.
11. Resuspend the pellet with the use of a micro-pipette in 1 ml of ice-cold 0.1M $CaCl_2$.
12. Freeze 80 µl of cells with 150 µl glycerol, at - 80 °C.

Annex V – Cells Transformation

1. Take competent cells out of -80°c and thaw on ice, for 30min.
2. Take agar plates, with the antibiotic, out of 4°C to warm up to room temperature.
3. Mix, gently, 50 ng of DNA into 80 µl of competent cells.
4. Place the mixture on ice for 30 min.
5. Heat shock each transformation tube by placing the tube into a 42 °C water bath for 45 sec.
6. Put the tubes back on ice for 2 min.
7. Add 300 mL of SOC medium, without antibiotic.
8. Grow for 1 h 30 min, at 37°C on a shaking incubator.
9. Plate the total volume of the transformation onto the LB agar plate.
10. Incubate plates at 37°C overnight.

Annex VI – Preparation of SDS-PAGE

To the SDS-PAGE, a gel of 12% of acrylamide was prepared with the components and amounts indicated on Table 23.

Table 23 – Components of SDS-PAGE

Components	12% Running Gel (mL)	4% Stacker Gel (mL)
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	3.5	0.4
0.5 M Tris-HCl (pH 6.8)	-	1
1.5 M Tris(pH 8.8)	3	-
H_2O	4.8	2.35
10% (w/v) SDS (sodium dodecyl sulfate)	0.12	0.04
TEMED (Tetramethylethylenediamine)	0.006	0.003
10% (w/v) APS (Ammonium Persulfate)	0.4	0.2

Annex VII – Prediction of Transmembrane Helices and Signal Peptides

In graphics below, the X-axes represent the amino acid position and the Y-axes show the probability that the amino acid is located within the membrane, periplasm or outside the cell, or in the cytoplasm and the presence or absence of the SP.

The results indicate the segments of the protein that lie in cytoplasmic region (green), non-cytoplasmic regions (blue) or within (grey) the membrane, and the SP (red).

Prediction of gi|30420959|gb|AY228551.1|

```
ID   gi|30420959|gb|AY228551.1|
FT   TOPO_DOM      1    343       NON CYTOPLASMIC.
//
```

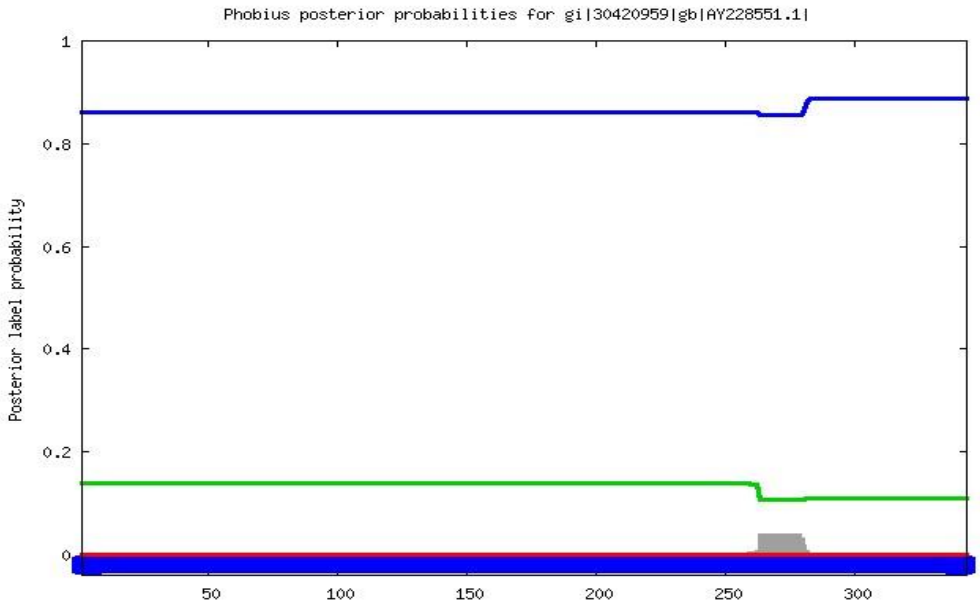


Figure 14- Identifying transmembrane domains and the signal peptides of dispersin B, using the *Phobius* database.

Prediction of gi|363539570|ref|YP_004893855.1|

```
ID   gi|363539570|ref|YP_004893855.1|
FT   TOPO_DOM      1    712       NON CYTOPLASMIC.
//
```

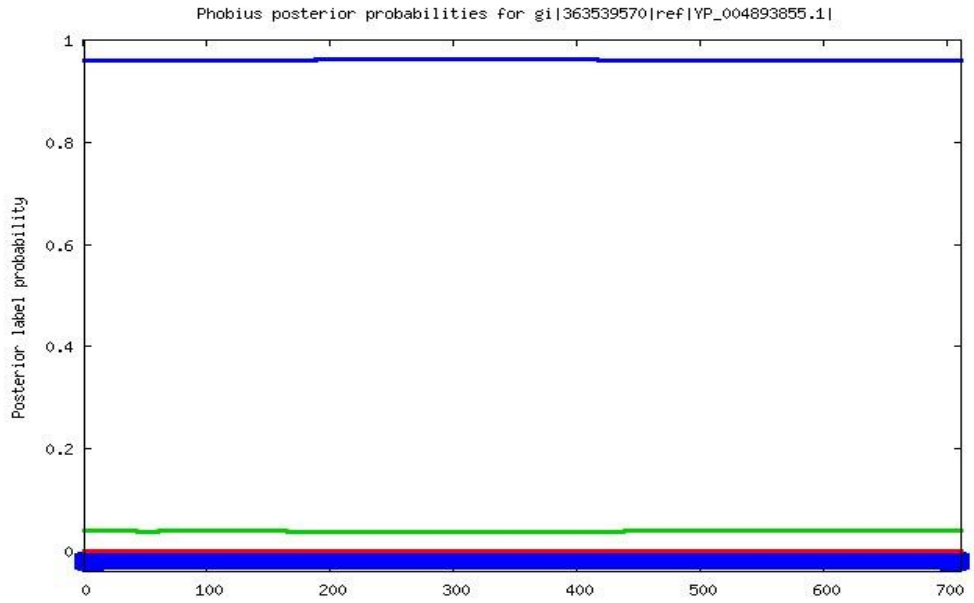


Figure 15 - Identifying transmembrane domains and the signal peptides of gp49, using the *Phobius* database.

Prediction of gi|363539667|ref|YP_004893952.1|

```
ID   gi|363539667|ref|YP_004893952.1|
FT   TOPO_DOM      1    236       NON CYTOPLASMIC.
//
```

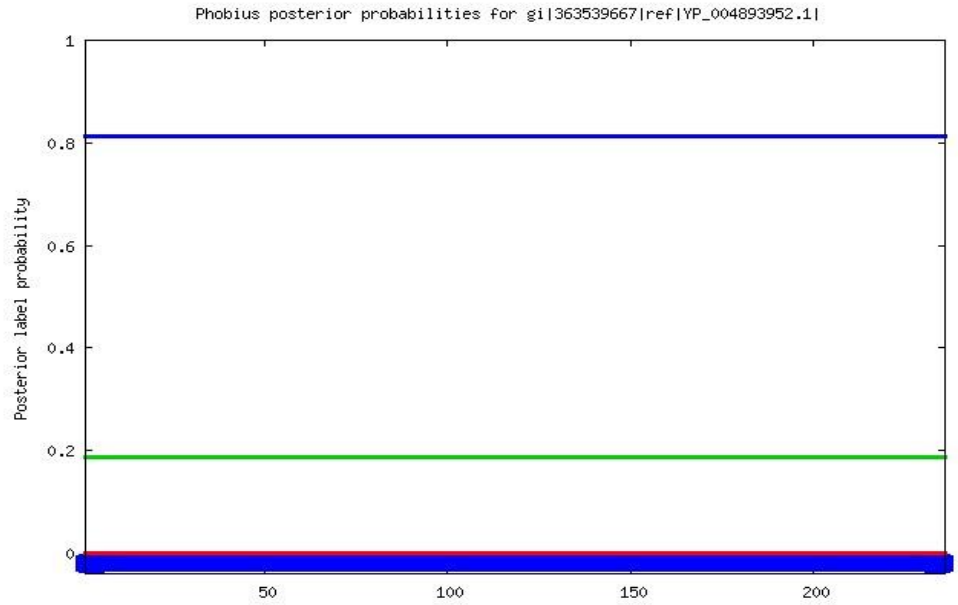


Figure 16- Identifying transmembrane domains and the signal peptides of gp146, using the *Phobius* database.

Prediction of gi|160380505|ref|YP_001551775.1|

```
ID   gi|160380505|ref|YP_001551775.1|
FT   TOPO_DOM      1      5       NON CYTOPLASMIC.
FT   TRANSMEM      6     26
FT   TOPO_DOM     27     37       CYTOPLASMIC.
FT   TRANSMEM     38     56
FT   TOPO_DOM     57     61       NON CYTOPLASMIC.
FT   TRANSMEM     62     81
FT   TOPO_DOM     82    105       CYTOPLASMIC.
//
```

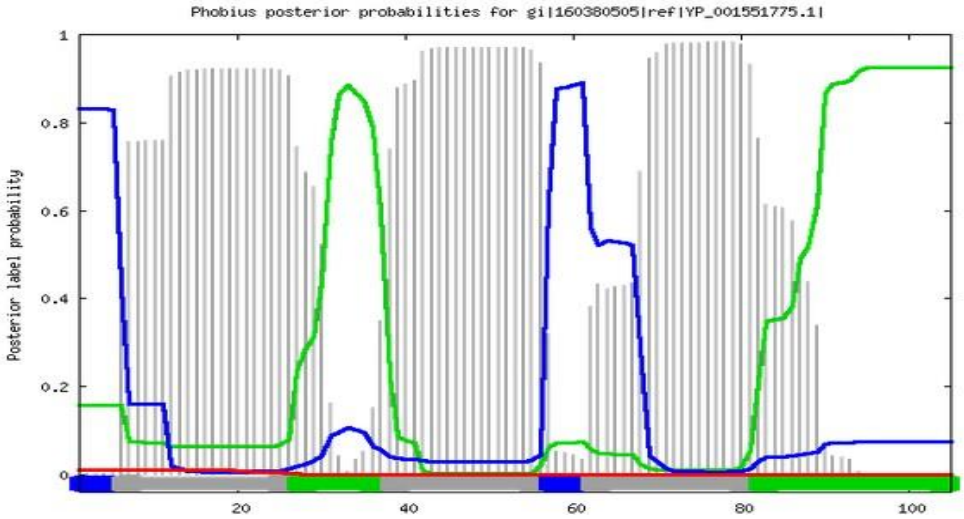


Figure 17 - Identifying transmembrane domains and the signal peptides of holin Lambda, using the *Phobius* database.

Prediction of gi|45686347|ref|YP_003932.1|

```
ID   gi|45686347|ref|YP_003932.1|
FT   SIGNAL        1     20
FT   REGION        1      3       N-REGION.
FT   REGION        4     15       H-REGION.
FT   REGION       16     20       C-REGION.
FT   TOPO_DOM     21     29       NON CYTOPLASMIC.
FT   TRANSMEM     30     47
FT   TOPO_DOM     48     71       CYTOPLASMIC.
//
```

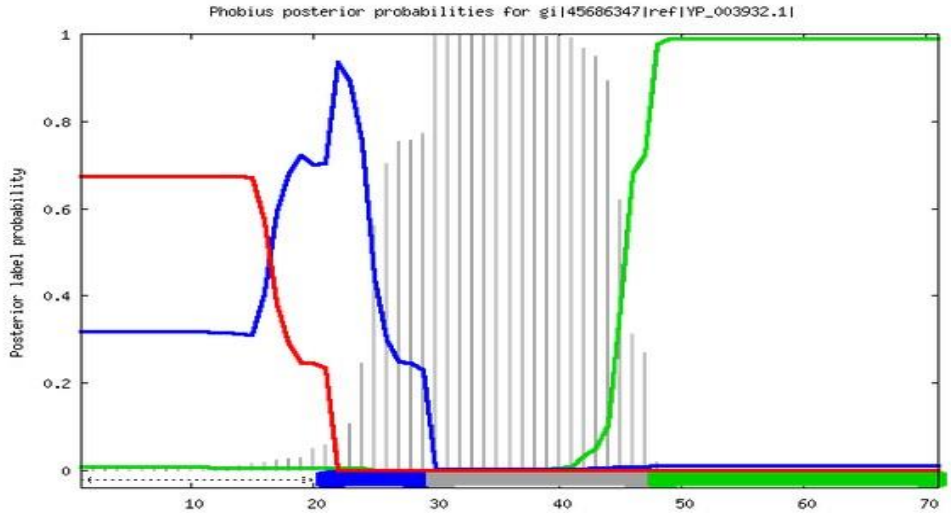


Figure 18 - Identifying transmembrane domains and the signal peptides of holin T1, using the *Phobius* database.

Prediction of gi|9632830|ref|NP_049865.1|

```
ID   gi|9632830|ref|NP_049865.1|
FT   TOPO_DOM      1     30       NON CYTOPLASMIC.
FT   TRANSMEM     31     49
FT   TOPO_DOM     50    218       CYTOPLASMIC.
//
```

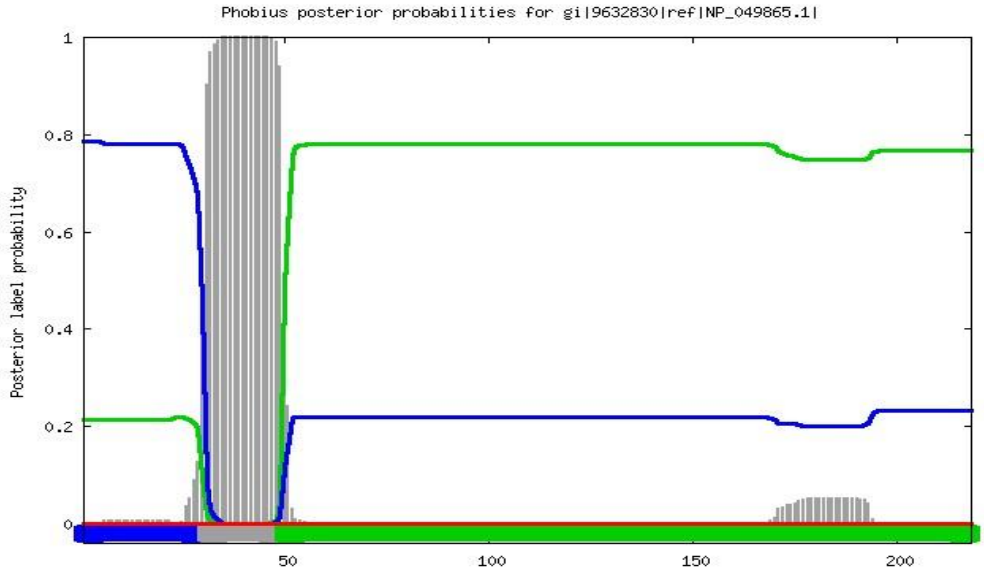


Figure 19 - Identifying transmembrane domains and the signal peptides of holin T4, using the *Phobius* database.

Prediction of gi|9627478|ref|NP_042006.1|

```
ID   gi|9627478|ref|NP_042006.1|
FT   TOPO_DOM      1     36       NON CYTOPLASMIC.
FT   TRANSMEM     37     55
FT   TOPO_DOM     56     67       CYTOPLASMIC.
//
```

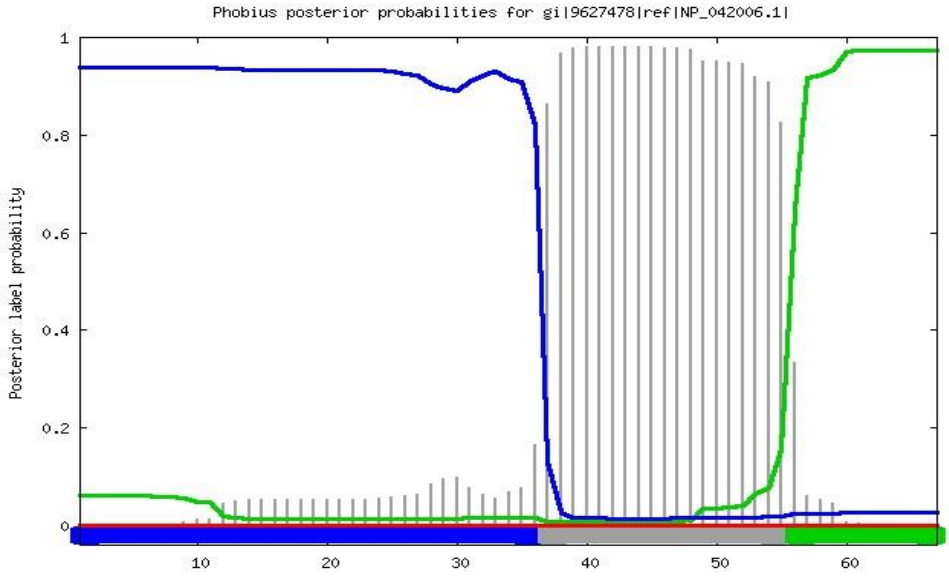


Figure 20 - Identifying transmembrane domains and the signal peptides of holin T7, using the *Phobius* database.

Prediction of gi|45686349|ref|YP_003934.1|

```
ID   gi|45686349|ref|YP_003934.1|
FT   SIGNAL        1     25
FT   REGION        1      5       N-REGION.
FT   REGION        6     17       H-REGION.
FT   REGION       18     25       C-REGION.
FT   TOPO_DOM     26    103       NON CYTOPLASMIC.
FT   TRANSMEM    104    125
FT   TOPO_DOM    126    133       CYTOPLASMIC.
//
```

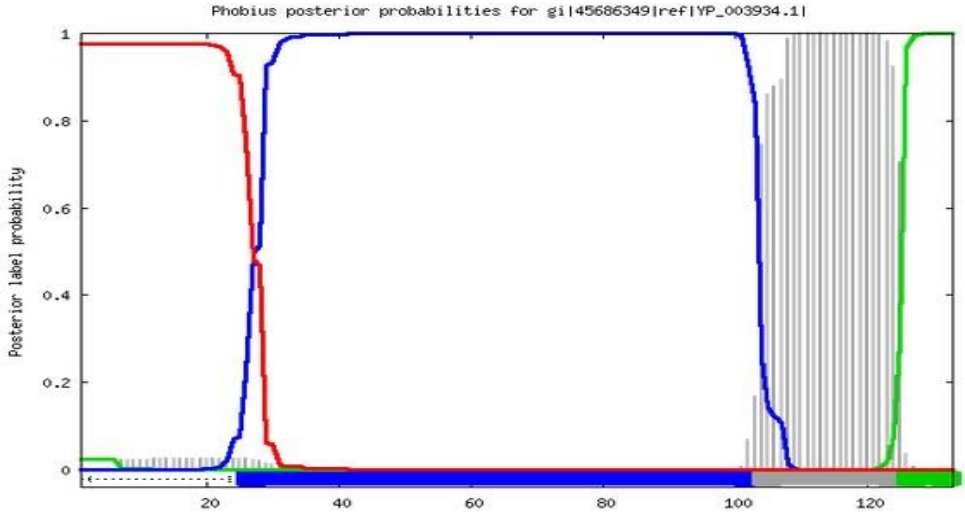


Figure 21 - Identifying transmembrane domains and the signal peptides of RzT1, using the *Phobius* database.

Prediction of gi|160338810|ref|YP_001551744.1|

```
ID   gi|160338810|ref|YP_001551744.1|
FT   SIGNAL        1     22
FT   REGION        1      7       N-REGION.
FT   REGION        8     18       H-REGION.
FT   REGION       19     22       C-REGION.
FT   TOPO_DOM     23     60       NON CYTOPLASMIC.
//
```

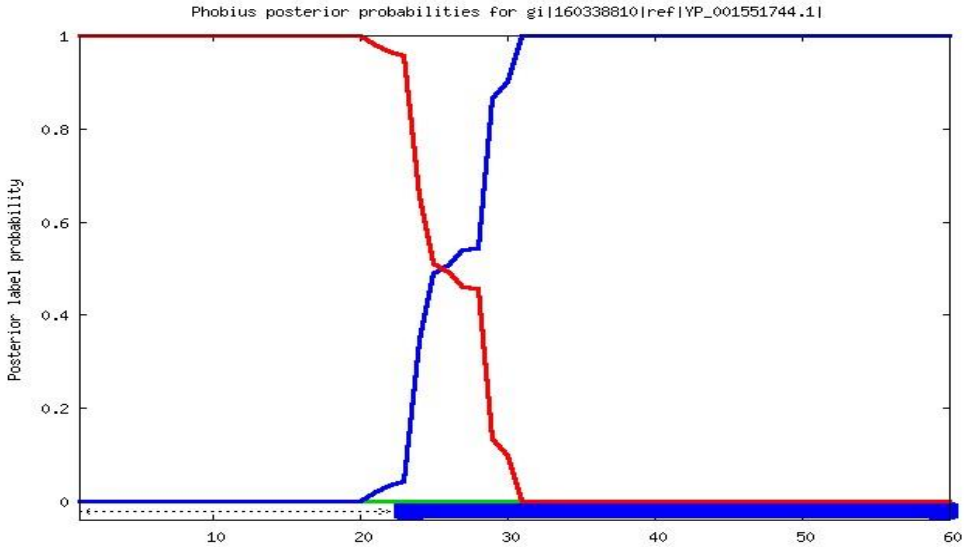


Figure 22 - Identifying transmembrane domains and the signal peptides of R1 Lambda, using the *Phobius* database.