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Escola de Engenharia

Ana Catarina Ribeiro Brandão

Fast detection of *Salmonella* based on viral peptides

Outubro de 2016

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Trabalho efetuado sob a orientação do

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ABSTRACT

Salmonella is a genus of Gram-negative bacteria that are widely distributed in nature, and most of which are associated with foodborne diseases and outbreaks. The high diversity and distribution in the environment, various routes of infection, survival capacity in several environmental conditions, and increased antibiotic resistant strains, make these bacteria a priority target for control and detection in the for-health care services, food industry, agriculture and livestock. However, the *Salmonella* detection is performed through traditional detection methods, which in turn suffer from various limitations, like the higher limits of detection, low sensitivity and specificity, are time consuming, unable to distinguish viable cells from nonviable cells, higher costs, time-consuming and have complicated procedures. The development of novel detection methods that overcome these difficulties is urgent, requiring the application of highly specific recognition agents, such as bacteriophages (phages), which are viruses capable of specifically infect bacterial host. The tail fibers proteins (TFP) from tailed phages (which are the most abundant phages) are responsible for phage attachment and therefore by the early specific bacterial host recognition. The application of the TFP as tool in methods is one of the most promising approaches for creating new detection methods.

In this work, it was identified and cloned genes from multivalent PVP-SE1 phage and PVP-SE2 that could encode TFP in fusion with aceGFP fusion partner, for posterior expression in *E. coli* (BL21 DE3) cells with and without chaperones from T4 phage or PVP-SE2 phage, using as a control approach the expression of gp37 and gp12 T4 TFP in the presence T4 chaperones, as described in the literature. From the cloned and expressed genes was identified only one TFP (the gp27 of PVP-SE2 phage) that recognized *Salmonella*, regardless of the chaperones use or not. The gp27 showed high specificity in *Salmonella* Enteritidis (one of the most prevalent serotypes) identification, and ability to identify 68% of serotypes from of *Salmonella enterica* subspecies I (highly broad and diverse group that includes most prevalent zoonotic *Salmonella* bacteria). Concluding, the TFP gp27 is a powerful tool for future applications in *Salmonella* high efficient-detection methods, and as adjuvant in *Salmonella* therapy due to its possible agglutination properties.

Keywords: *Salmonella*, Detection methods, Bacteriophages, TFP, Chaperones.

RESUMO

Salmonela consiste num género de bactérias Gram-negativas que se encontram amplamente distribuídas pela natureza, e das quais a maioria está associada a doenças e surtos de origem alimentar. A elevada diversidade e distribuição no meio ambiente, variadas vias de infeção, capacidade de sobrevivência face a várias condições ambientais, e aumento de estirpes resistentes a antibióticos, fazem destas bactérias um alvo de controlo e deteção prioritário para os serviços de saúde, indústria alimentar, agricultura e pecuária. Contudo, a deteção de *Salmonella* é efetuada através de métodos de deteção tradicionais, que por sua vez sofrem de variadas limitações, desde os elevados limites de deteção, baixa sensibilidade e especificidade, são morosos, incapacidade de distinguir células viáveis de células não viáveis, altos custos, morosos e com procedimentos complicados. O desenvolvimento de novos métodos de deteção que ultrapassem estas dificuldades torna-se urgente, sendo necessário a aplicação de agentes de reconhecimento altamente específicos, como por exemplo os bacteriófagos (fagos), que são vírus com capacidade de infetar especificamente bactérias. As proteínas presentes nas extremidades das fibras de fagos com cauda ("*Tail fiber proteins*" -TFP) são as responsáveis pela adsorção do fago à bactéria e, portanto, pelo reconhecimento específico da bactéria hospedeira. A aplicação das TFP como ferramenta de deteção é umas das abordagens mais promissoras para a criação de novos métodos de deteção.

Neste trabalho foram identificados e clonados genes do fago multivalente PVP-SE1 e fago PVP-SE2 que possam codificar TFP, com o parceiro de fusão aceGFP, de modo a expressar em células *E. coli* BL21 (DE3) com e sem chaperones do fago T4 e fago PVP-SE2, usando como abordagem controlo (já descrita na literatura) a expressão das TFP gp37 e gp12 de fago T4 na presença de chaperones também do fago T4. Dos genes clonados e expressados apenas foi identificada uma TFP (gp27 do fago PVP-SE2) capaz de reconhecer *Salmonella,* independentemente do uso ou não de chaperones. A gp27 demonstrou elevada especificidade na identificação de *Salmonella* Enteritidis (um dos serotipos mais prevalentes), assim como capacidade de identificação de 68% de serotipos da subespécie I de *Salmonella enterica* (grupo altamente alargado e diversificado que inclui a maioria das bactérias de *Salmonella* zoonóticas e prevalentes). Em suma, a TFP gp27 constitui uma poderosa ferramenta com futuras aplicações em métodos de deteção de *Salmonella* com alta eficiência, e adjuvante na terapia de *Salmonella* devido às propriedades aglutinantes que possa apresentar.

Palavras-chave: *Salmonella*, Métodos de deteção, Bacteriófagos, TFP, Chaperones.

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List of abbreviations and Acronyms

AOAC	Official Analytical Chemists
aceGFP	Green Fluorescent Protein
AK	Adenylate kinase
APS	Ammonium persulfate
ATP	Adenosine tri-phosphate
BEIA	Bioluminescent enzyme immunoassay
BIND	Bacterial Ice Nucleation Detection
BSA	bovine serum albumin
CBDs	Cell Wall Binding Domains
CDC	Centers for Control and Disease
CFU	Colony-forming unit
CPEC	Circular Polymerase Extension Cloning
DEFT	Direct fluorescent filter technique
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EIA	Enzyme immunoassay
ELFA	Enzyme-linked fluorescent t assay
ELISA	Enzyme-linked immunosorbent assay
EMA	Etidium monoazide
EU	European Union
FDA	Food and Drug Administration
FISH	*In situ* Hybridization
GMOs	Genetically Modified Organisms
gp	Gene product
HACCP	Hazard Analysis and Critical Control Point
ICTV	International Committee on Taxonomy of Viruses
IGG	Immuno-chromatography
IMS	Immunomagnetic separation

inaW	Bacterial ice nucleation gene
IPTG	Isopropyl-β-D-1- thiogalactopyranoside
LacZ	β- Galactosidase expressing gene
LB	Lysogenic Broth
LFD	Lateral flow devices
LPS	Lipopolysaccharide
lux	Luciferase expressing gene
MCS	Multiple Cloning Sites
MEMES	Microeletromechanical
mRNA	Messenger ribonucleic acid
O.D	Optic density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PMA	Propidium monoazide
PNA	Peptide nucleic acids
QDs	Fluorescent Quantum Dots
qPCR	Real-time PCR
RBPs	Receptor Binding Proteins
RIA	Radio-immunoassays
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS	Sodium Dodecyl Sulfate
spp.	Species
SPR	Surface Plasmon resonance
subsp.	Subspecies
TAE	Tris-acetate-EDTA
TFP	Tail fiber proteins
TG	Tris-glycine buffer
TGS	Tris-glycine-SDS buffer
TMED	Tetramethylethylenediamine
tRNA	Transfer ribonucleic acid

VBNC	Viable but non-culturable cells
v-PCR	Viability PCR
WHO	World Health Organization
ZsYellow	Yellow fluorescent protein encoding gene

Chapter 1

Introduction

1.1 *Salmonella* spp. as a foodborne pathogen

Salmonella is a complex genus of Gram-negative, facultative anaerobic, rod-shaped, non-spore forming bacilli and flagellated bacteria, classified as oxidase-positive and catalase-negative bacteria. Their survival occurs in a temperature range of 5.1 - 46.2 °C, and a pH range of 3.8 - 9.5, being able to grow in meat extract media and use several sugars with or without gas production [1–3].

Salmonella genus is a member of the *Enterobacteriaceae* family and is constituted by two species: *Salmonella bongori* and *Salmonella enterica*, this last one is the most known and comprises most of the *Salmonella* strains. *Salmonella enterica* is composed by six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae and indica)* that are further subdivided into serotypes according to their flagellar, carbohydrate and lipopolysaccharide (LPS) structures [1,2,4,5].

Some of these bacteria have only one host, but the majority have a wide variety belonging to a vast group of pathogens that infects humans and animals, considered thus as zoonotic [6,7]. Indeed, most of these pathogens belongs to the *S. enterica* subsp. *enterica,* that includes more than 1 500 serotypes, responsible for 99% of the infections in humans and animals [3,8]. Each serotype is characterized according to their host specificity (or geographical regions), by the clinical syndrome that it causes, potential antimicrobial resistance and by their antigenic presentation [5,9,10]. Namely, *S. enterica* subsp. *enterica* serotypes can be classified as typhoid (for example *S.* Typhi and *S.* Paratyphi serotypes) or non-typhoid serotypes (of which *S.* Typhimurium and *S.* Enteritidis are the most common described serotypes associated with foodborne illness). The typhoid *Salmonella* serotypes are causative agents of enteric fever which is a human-limited disease, endemic, life-threatening and systemic disease, typical of undeveloped regions where there is no basic conditions [3,6,8,9,11]. On the contrary, the non-typhoid *Salmonella* serotypes are founded globally and have the capability to infect several hosts, being responsible for million cases of gastroenteritis globally each year [5,8,12,13].

Thus, *Salmonella* bacteria are recognized to be the cause of several types of human illness, including systemic infections (mainly in immune-compromised people and in children), fever, enterocolitis/diarrhea, nausea, abdominal pain, bacteremia, asymptomatic carriage, vomiting and sometimes septicemia. However, salmonellosis usually are a self-limited infection and does not demand antibiotic treatment [2,8,14–16].

The *Salmonella* bacteria is one of the main foodborne pathogens worldwide, being responsible in European Union (EU) for about 100 000 of salmonellosis cases in humans and an economic burden for the health care systems that can be higher than 3 billion euros *per* year [17,18]. Also in USA, the Centers

for Disease control and Prevention (CDC) estimates that *Salmonella* are the cause of one million foodborne illnesses in the United States, with 19 000 hospitalizations and 380 deaths every year [19].

To reduce the salmonellosis cases and to assure the consumers safety, it was developed a surveilling program that aims to establish the safety along the entire food supply chain by the World Health Organization (WHO) and associated organizations [20]. The coordinated approach applied by the responsible institutes of zoonotic diseases has decreased the human cases of salmonellosis by the establishment of good agricultural and manufacture practices, hygiene and quality control by hazard analysis and critical control point (HACCP), and application of well-designed biocides and sanitizers. However the number of outbreaks remains to be huge due to the continuously appearance of pathogens in food and food contact surfaces, which is still a major concern for public health, animal health, agricultural and food industry [6,16,21–23].

Apart from the large *Salmonella* incidence and outbreaks number, these bacteria are able to resist to a several types of environments, presenting a vast distribution in nature (being present in water, plants, soil, animals and human intestine) (Figure 1) [7,8,22,24]. Therefore these pathogens easily infect their hosts (which consists in a broad spectrum) and produces serious illness, which in humans are generally initiated by the ingestion of contaminated water and food (raw/undercooked eggs, poultry and meat, raw milk and dairy products, seafood, salad, vegetables, fruits, among others) [9,10,25,26]. Thereafter, the bacteria passes from the low gastric tract to the intestinal epithelium and subsequently colonize the mesenteric lymph nodes and internal organs in the case of systemic infections [10,27–29].

The main reservoirs of this pathogen are the domestic animals (poultry, cattle, swine and sheep) [7,30] which are directly related to food contamination, particularly in agriculture, when the feces of the animals are used as fertilizing. Consequently the obtained products, water courses (that are used for irrigation), and soil become contaminated [8,10,31]. Food can also be contaminated during the manufacture or storage, due to bad cleaning practices and inadequate temperatures. Thus, *Salmonella* can be transmitted by fecal-oral route, and person-to person or pets/wild animals- contact [8,9].

The incorrect employment of antibiotics as growth promoters and therapeutic agents in animal feeds, health care systems and veterinary made for several years, has been also a concern related to *Salmonella* bacteria, since it has been observed an associated increasing of appearance of some multidrug-resistant *Salmonella* serotypes worldwide, complicating and diminishing the possibility to treat patients with acute infections and reduce the outbreaks [9,14,32].

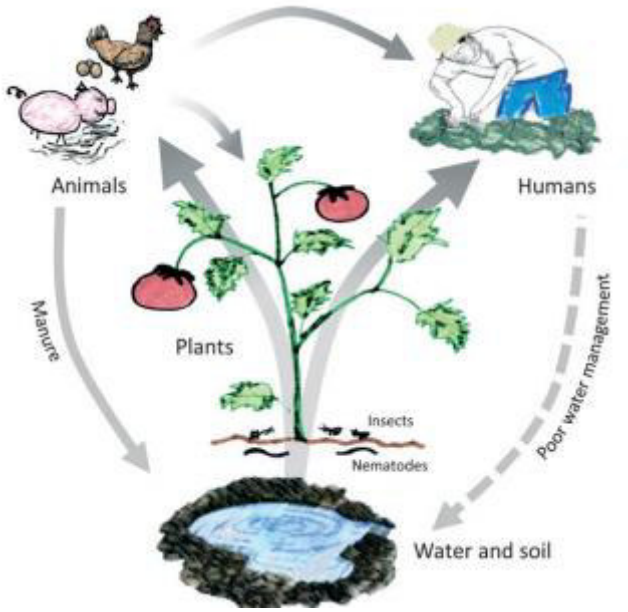

Figure 1- Infection routes and main reservoirs of *Salmonella* in ecosystem [24].

To prevent a dreadful effect, the application of rapid and early detection methods able to monitor *Salmonella* in the soil, water reservoirs, hospitals, food products, animals feed and farms (infected animals and cultures) is needed in order to assure the animals and humans health safety [33]. However, *Salmonella* control is a challenge due to its ability to infect several host types, the increase of antibiotic resistance capability, its higher incidence, and the easy way of transmission and infection [34].

Since this problem is an important and urgent subject for health care and food industry, and since the current efforts and proceedings are not enough to avoid the occurrence of many infections, new procedures to overcome antibiotics as therapeutic procedure, and the slow turnaround of classic methods of pathogen detection must be created to detect and control/eradicate *Salmonella* bacteria.

1.2 Conventional methods for *Salmonella* detection

The prevention of outbreaks associated with *Salmonella s*pp. and others foodborne pathogens rely in their early identification and detection in contaminated food [16]. To accomplish this, there are several available methods, which include the culture and colony methods, the polymerase chain reaction (PCR), immunological based methods, nucleic acid probes, biosensors and electrochemistry [12,18,21,26,35].

These traditional methods are well described and their application has been approved for a very long time satisfying some requirements in the pathogen detection, but their limitations contributes for a poor evaluation of foodborne pathogens presence [12], which creates the necessity to develop new procedures and techniques in the food industry that can be more fast, reliable, specific, selective, stable, economic, *in situ* devices, with real time assays, capable to detect small quantities of the target microorganism (*Salmonella* infection dose is 15-20 cells), and minimizing the false positives and negatives [12,21,26,36].

1.2.1 Culture and colony based methods

The golden standard for *Salmonella* detection is the standard culture method according to ISO 6579: 2003 (Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp.), especially in regulatory agencies, since it remains the most reliable, accurate, well-accepted and harmonized method [21,37,38]. This technique is based on the microorganism's capacity to grow and multiply in solid media, generating visible colonies at the naked eye. Usually, consists in four stages: a non-selective pre-enrichment in liquid medium, selective enrichment in liquid medium, plating in differential and selective agars, and finally, the serological and biochemical identification of suspected colonies (Figure 2) [33,38,39].

The biggest advantage of this method is that, theoretically, the obtained results correspond only to viable-cells while the growth of remaining organisms is suppressed. However, during the cultural enrichment, the presence of other competing organisms can diminish the specificity and sensitivity of agar media, and consequently it is obtained a high quantity of false positives. The sensitivity can also be affected by the use of antibiotics, inadequate sampling and small number of viable cells in samples [38].

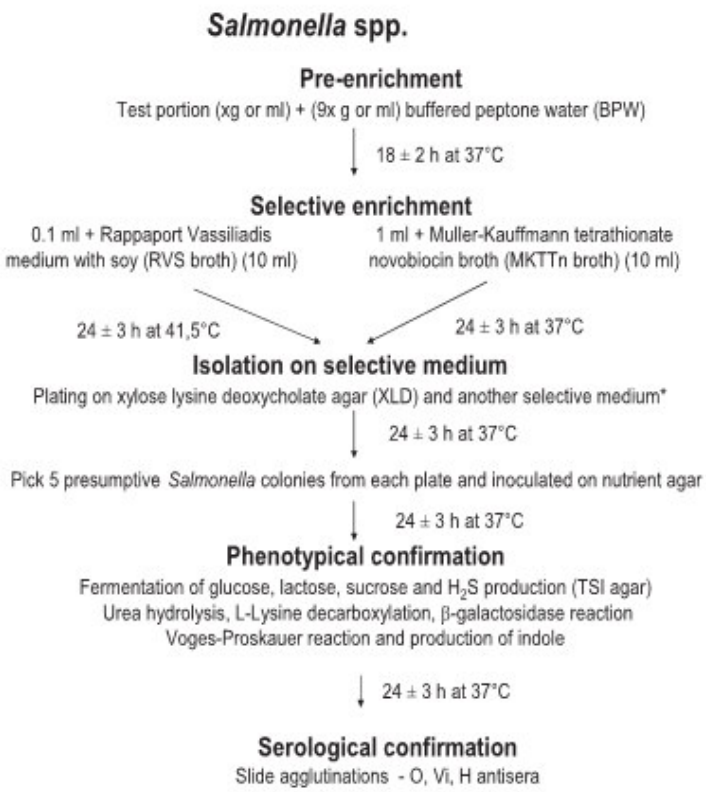

Figure 2- Main steps of *Salmonella* detection through Culture and Colony technique [39].

In food industry, the several treatments and processing's that are made to the food can damage the *Salmonella* viable cells, which might be related to cells entrance into a dormancy state and become non-cultivable or unable to originate visible macroscopically colonies in solid medium. These damaged cells posteriorly can repair themselves and proliferate during the food storage product, being an additional problem that leads to a bad quality product evaluation. These cells, viable but non-cultivable cells (VBNC), have been reported for various pathogenic bacteria as a result to its submission to stress conditions, for example, the disinfection treatments [21,25]

Others drawbacks can be pointed to the culture based methods including being time-consuming (up to 7 days, 3-4 days for initial results and 7-10 days for confirmation), labor intensive and complex method (complicated sample preparation that requires specialized operators to perform it), expensive (demands for large volumes of liquid and solid media/reagents), limiting its application on food industry [21,33,37,39,40]. Indeed, these methods are designed for laboratory use, not being expected to adapt in the field [33]. The classical culture methods have a detection limit of about 4 CFU/mL for liquid foods and 4 CFU/g for solid foods according some reports [39] or 1 cell/25 g for other reports [41].

1.2.2 Immunoassays

The immunological techniques are all based on the highly specific binding reaction between antibodies and somatic or flagella antigens from bacteria surface. These methods include Enzyme-linked immunosorbent (ELISA) and fluorescent (ELFAs) assays, latex agglutination tests, immunodiffusion, Immuno-magnetic separation (IMS), lateral flow devices (LFD) and immuno-chromatography (IGG) strip test, enzyme immunoassay (EIA), bioluminescent enzyme immunoassay (BEIA), immunoprecipitation assay, radio-immunoassays (RIA), western blot, among others [16,21,39].

The most common immunological methods applied in pathogen detection are ELISA and ELFA, which have in common the sandwich assay that rely in the immobilization of a specific antibody in a solid matrix surface of a 96-well microtiter plate, following by sample addition (to promote the specific binding between the antibody and the bacteria somatic/flagellar antigens) forming an antigen-antibody complex. The non-binding bacteria are washed and finally the complex formation can be then measured by an enzymatic reaction that produces an alteration of color or by fluorescence, due to the use of a secondary antibody labelled with an enzyme or a fluorescent component, or other variants (Figure 3) [39,42,43].

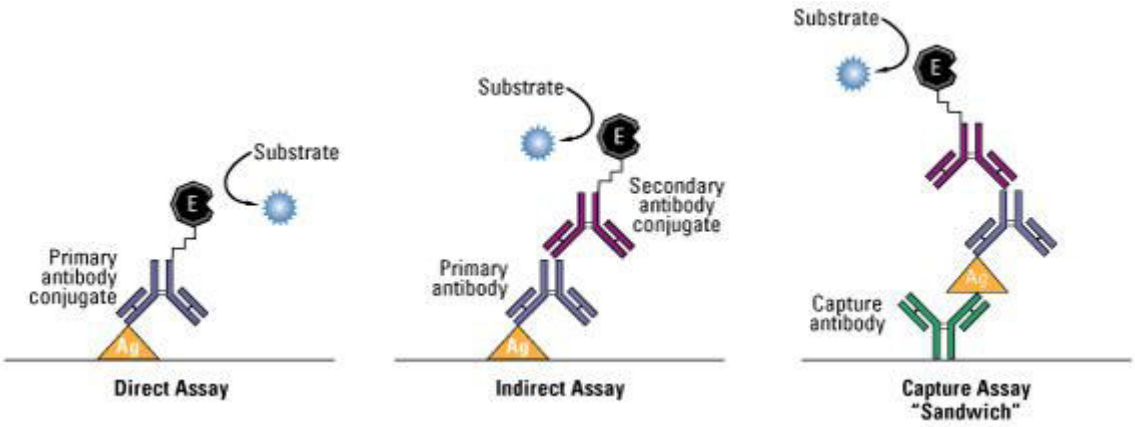

Figure 3- Different common procedures to perform ELISA (available in https://www.thermofisher.com).

The latex agglutination assay consists in the use of latex particles coated with antibodies that react with *Salmonella* antigens, creating visible aggregates and allowing the identification of samples contaminated with *Salmonella* spp. [44,45]. This method is specific, reliable and simple, so it can be used both as a detection or confirmation method. The available kits in the market are Spectate (May & Baker Diagnostics Ltd., Glasgow, Scotland, UK), Wellcolex color *Salmonella* (Wellcolex, Merseyside, UK), *Salmonella* Latex test (Oxoid, Basingstoke, UK), Bactigen (Wampole Laboratories, Cranbury, NJ), and Slidex (Bio- Merieux, Marcy L'Etoile, France) [16].

The LFD is a technique that employs a porous membrane with immobilized protein as bio-recognition element to capture the bacteria. When the attachment occurs it is generated a signal due to the presence of gold nanoparticles or colored latex particles [39]. This assay is used for fist on-site plant pathogen and mycotoxin detection, although, it is time-consuming, with limited sensitivity, and it is not regenerated [46].

In the immuno-chromatography assay are made a pre-enrichment and selective enrichment steps, and then the sample is applied into the device and *Salmonella* cells are captured onto the dipstick. The commercial available dipstick tests for *Salmonella detection* are Tecra® *Salmonella* Unique™ test (Tecra International Pty Ltd, French Forest, New South Wales, Australia) and the PATH-STIK (Celsis, Inc., Edison, NJ) that only take 30 min of analysis [16]. Some of the assays rely in the assumption that 97.7% of *Salmonella* bacteria are hydrogen sulfide productive and are all enzyme C8 esterase productive, and combining the detection of this two components it was achieved a detection limit of 10^1 CFU/mL of *Salmonella* in the presence of 10^5 CFU/mL of *E. coli* [47].

Although the IMS assays methods are not considered a detection method, they have been applied as a powerful sample preparation tool, reducing the time and labor problems associated to selective enrichment steps before the detection itself, allowing the handle of large samples amounts [39,42]. In IMS, the superparamagnetic particles are coated with antibodies that specifically capture and isolate the bacteria cells from complex samples suspensions without inhibition from sample components or non-target microorganisms [23]. This technique is commercially available by Pathatrix® Auto System (Life Technologies, Grand Island, NY), and is already approved by Association of Official Analytical Chemists (AOAC), being applied to capture and concentrate bacteria from a variety of complex biological media, like food, faeces, and water environmental samples [28,48].

In general, the Immunoassays are classified as robust, fast (compared with culture methods) and sensitive methods that beyond the target pathogen cells can measure their bio-toxins too, and they can be automated which improves its efficiency (higher standardization and reproducibility) reducing the human errors and hands-on time [12]. However, the immunoassays performance depends on the appropriate antibodies (polyclonal or monoclonal) selection, the available ones have scarce variability, have limited shelf-life and its stability depends on the pH and temperature conditions, its specificity can be affected by cross-reactions with similar antigens, and have high costs. Additionally, the immunoassays sensitivity can be affected by some components from the sample matrix requiring skilled technicians, the processing sample volume is low and the assays need long enrichment times to obtain the appropriate number of cells increasing the required assay time [21,39,40,49–51].

The detection limits normally rounds the 10^4-10^5 CFU/mL and several commercial available devices exist like BIO A.R.T, R-BiopharmAG, BioControl systems, Rayal, 3M and VIDAS (Bio-Mérieoux) [38,39,52]. The rapid detection method VIDAS® Easy SLM it is also approved by AOAC for pathogen screening in foods with a detection limit of 0.2-2 CFU/25 g within 2 days [53].

1.2.3 PCR based detection methods

The PCR methods have been extensively used in the detection of foodborne pathogens, since they rely in a simple technique that amplifies small quantities of DNA template into multiple copies, with high specificity, sensitivity, speed, accuracy, and can be used as an automated diagnosis [54]. The most known variants are quantitative real-time PCR (qPCR), reverse transcriptase PCR (RT-PCR), Multiplex-PCR (which permits the simultaneously detection of various microorganisms in a single reaction), multiplex RT-PCR and real-time RT-PCR. These methods are used for *Salmonella* detection by targeting specific genes from *Salmonella* spp. that codes for enzymes, toxins, rRNA genes, and repetitive elements, like for example, the *invA* gene (involved in bacteria invasion), *ttr*RSBCA locus, *fim*C and *ori*C [1,13,21,38,40,55].

Despite the main advantages of these methods, they have several limitations that compromises the pathogen detection. In the first place the PCR associated rapidity and high sensitivity (detection of a few molecules) is always affected by the pre-enrichment that is necessary to obtain enough pathogen quantity, above the limit of detection (10^3-10^4), which is still too high for *Salmonella* detection (should be absence in 25 g of food sample) [28,35,54,56,57]. Also, from an industrial point of view, pathogens detection through PCR can be expensive, the method is not adapted for field applications, and it requires skilled technicians to carry out it [21,28,37,40,52].

The pathogen detection made by PCR cannot distinguish if the DNA is from viable or non-viable bacteria, once the DNA from non-viable cells remains stable even after the bacteria death, and this limitation is responsible for the high false positives associated to PCR [36]. Therefore, the inclusion of suitable controls for pathogens detection in food samples by PCR is crucial. Indeed, from the several reported PCR detection methods, many of them do not include an internal amplicon control, being based in nonspecific melting curves analysis and their accuracy/selectivity is not rigorously tested [13]. Nevertheless, the false positives can be excluded by using real time RT-PCR that only amplifies cDNA converted from mRNA (a molecule that has a half-life of about 3-5 min after cell death) present in the sample, but for that it is required the expression of the target gene by the bacteria which can varies under stress conditions [21,25,36].

A possible alternative is the viability PCR (v-PCR) or the viability qPCR (v-qPCR), based in the detection of DNA from viable cells (with intact cell wall and /or membranes). This technique is based in a previous discrimination of viable cells, using EMA (etidium monoazide) and PMA (propidium monoazide) that can enter in damaged cells and intercalate in their DNA (every 4-5 nucleotides), diminishing the DNA extraction. In viable cells there is no effect since these molecules are positively charged and only enter in negative charged (damaged) cells, being possible to extract DNA that posteriorly is amplified by qPCR [25,36,37]. However, some reports show that this approach is not completely efficient in the discrimination between viable and non-viable cells, still leading to an overestimation of bacterial number present in samples (false positives) [36,58–60].

The PCR can also be limited by the presence of inhibitory components like, food components, humid acid, urine, bile salts and others [39,54]. The removal of these components involves additional filtration, concentration and IMS steps before the DNA extraction which makes it a laborious method [54]. The PCR can also be affected by the contamination with other bacterial DNA extracted from the sample that may in turn increase the false-negative results, making necessary the posterior utilization of an electrophoresis or heterogeneous hybridization to eliminate the carryover risks [38].

Validated PCR methods are available from Bio-Rad, Roche, Qualicon/Oxoid, Genesystems, AES Chemunex, Applied BioSystems, Idaho Technology Inc., Lantmännen, IEH Laboratries and Consulting Group, ADNucleis and BioControl systems [61].

The microarrays technology is a technique that allows the detection of multiple organism's by hybridization of fluorescent-labelled single strand nucleotide chains to an array of nucleotide immobilized probes into a glass slide, using PCR amplification prior to hybridization to increase the sensitivity of detection [23,39]. The major microarray drawback is the food matrix complexity, and the analysis of the high quantity generated data. Additionally, contrary to the reported reduced cost for the multiple bacteria analysis [38,62], this technique is still very expensive [63,64]. The commercial Premi® Test *Salmonella,* is based in microarray system and is used for most common *Salmonella* enterica serovars detection with results within 8 hours [39].

1.2.4 Nucleic acid based detection methods

The nucleic acid hybridization includes fluorescence *in situ* hybridization (FISH), DNA hybridization and sandwich hybridization, and has the advantage that it only detects viable cells.

The FISH method consists in hybridization of 16S rRNA oligonucleotide probes labelled fluorescently with the rRNA sequence from ribosomes of immobilized cells (previously prepared and permeabilized). Then the unbound probe is washed and the preparation is visualized under the fluorescent microscope [38,39,43,65]. The detection of *Salmonella* in food with this method seems to be very sensitive (due to the use of RNA probes instead of DNA), fast and cheap, and this is due to the fact of rRNA sequences being highly conserved, stable, and abundant in a bacteria cell, providing various targets and increasing the assay sensitivity, allowing too the detection of VBNC cells. The reported detection limit is 10^4 CFU/mL and the time usually needed is about 3h after an overnight enrichment, and an example of an available commercial assay is Vermicon [38,39]. *Almeida et al*. developed a new FISH technique that involves the use of peptide nucleic acids (PNA) for the specific recognition of *Salmonella* with high sensitivity and specificity [66]. This synthetic peptide shares complementarity with a conserved RNA-host sequence, being able to detect 1 CFU/10 mL in less than 24 h [66,67].

In the DNA hybridization assay, the DNA from samples is incubated with a DNA probe which has the DNA/RNA complementary sequence to the molecule in the target organism (enzymes and toxin encoding genes, repetitive elements). The probe that does not hybridize is washed, and stable hybrids with labelled DNA are detected by radioisotope, fluorescent or enzymatic reaction. The advantage of DNA hybridization is its highest specificity and sensitivity in the identification of *Salmonella* compared to other methods. However, the required sensitivity may be achieved only in the presence of sufficient concentration of target organisms after the pre-enrichment [68].

The fluorescent probes can be visualized under the epi-fluorescent microscope or by flow cytometry in combination with live-death straining. Indeed, the flow cytometry has already been applied in milk analysis using ethidium bromide to stain bacteria present in the sample, and it was used too to analyze the samples from water, beverages or dairy industries. The fluorescent microscope can be used by applying the Direct Fluorescent Filter Technique (DEFT) that consists in bacterial concentration and collection into a membrane and posterior staining with dyes and direct filter-surface visualization. The method can be automated by linking the microscope to an image analyzing system. The detection limit for both flow cytometry and fluorescent microscopy analysis rounds the 10^4-10^5 cells/mL [39] or 10^2-10^3 cells/mL [68].

1.2.5 Alternative detection methods

i. Biosensors

The biosensors are tools that consists in the immobilization of bio-receptors or bio-recognition elements in the surface of a physicochemical transducer, which generate a specific recognition signal when a biological component binds to the bio-receptor. The signal can be the change of mass, oxygen consumption, potential difference, refractive index, pH variation, current, and other parameters, being proportional to the biological response from the bioreceptor. The biological recognition elements used in biosensors include enzymes, antibodies, nucleic acids, organelles, whole cells, tissue/whole organisms, molecular imprinted polymers, bacteriophages (and phage peptides) and biomimetic material. The signal of recognition and their transduction in the biosensor is achieved by different types of transducers: electrochemical, optical, thermometric, piezoelectric, magnetic, micromechanical, mass based, and other miscellaneous transducers. The minimal input signal from the transducer is amplified with a large output signal that is therefore processed and analyzed [21,33,39,40].

The majority of biosensors rely in the application of antibodies and nucleic acids, but both suffer from drawbacks as referred in the previous sections [26,33,69]. The main advantages of biosensors include their sensitivity, cost-effectiveness, miniaturization and portability, automation, rapidness, easy of operation, real time detection, lower detection limits, and the absence of a pre-enrichment step. However, they suffer from one important drawback: the inclusion of false-negatives, *i.e.* the failure to detect the pathogen presence in the sample. The appearance of these false-negatives is connected to the low sensitivity, matrix interferences and inhibitors and the capacity of bacteria to enter in a “dormant” state (recovering in favorable conditions and manifesting their virulence) [25,26,33,35,40].

Also, these devices work with small sample volumes (nanoliters) that difficult the handling of large volume samples required for food and environmental testing [70].

1.3 Bacteriophages

1.3.1 History

The discovery of bacteriophages (or phages) occurred in the end of 19th century, namely by Earnest Hankin in 1896 and Frederick Twort in 1915 [26,40,71]. In their findings, both have mentioned the bacteriophages ability to kill bacteria and to pass through Millipore filters that normally retain bacteria. In 1916 Felix D'Hérelle has made the same findings, but contrary to Twort, D'Hérelle investigated the nature of bacteriophages and their capacity to act as therapeutic agents against bacterial derived diseases. He was the one who suggested that bacteriophages are viruses and defined it "bacteriophages". Also, he established phage therapy centers in many countries, after the determination of the safety of its oral and subcutaneous-administration of phage preparations. Early trials using bacteriophage-based products were done and showed promising results although, due to the lack of knowledge about bacteriophages and the discovery of the penicillin antibiotic, the phage therapy was disused by the western countries [26,71].

1.3.2 Biology and classification

Bacteriophages are obligate intracellular viruses (with a size that rounds between 20-200 nm) that specifically infects its bacterial host, using the cell machinery for their own multiplication and dissemination [15,40]. They are the most simple and abundant entities on Earth being harmless to humans, plants, and animals, with an estimated number of global phage population of 10^{30}-10^{32}. They are present wherever their bacterial host(s) inhabit, which consists in a wide range of habitats (soil, water, sewage, human and animal organisms, and even food). However, they are found predominantly in aquatic systems in a range of 10^{4}-10^{8} viruses/mL. The impact of bacteriophages in ecology is very important since they regulate the biogeochemical global cycles, being responsible for killing about 20% of prokaryotic microorganisms produced every day, and because they are a high diverse group of entities that are able to infect from only one specific strain or specie to many species and rarely an entire genera (the last ones considered to be polyvalent phages) [15,22,26,35,40,70,71].

Phages are composed by a nucleic acid molecule (double stranded-DNA, single stranded-DNA, double stranded-RNA or single stranded-RNA) inside of a protective capsid composed by subunits of proteins that surrounds the genome. Their classification is made by the International Committee on

Taxonomy of Viruses (ICTV), according to their morphology (size and shape), the genetic material, life cycle, host, proteomic and genomic analysis [15,26,70,71].

Morphologically, bacteriophages can be tailed viruses, polyhedral, filamentous or pleomorphic (Figure 4). The majority of the phages are dsDNA tailed phages (96%) with icosahedral heads, that belong to the *Caudovirales* order, which is divided in three families: the *Myoviridae* (with long, rigid, and contractile tails), the *Siphoviridae* (with long, flexible, and non-contractile tails) and the *Podoviridae* (with short and non-contractile tails) [26,70].

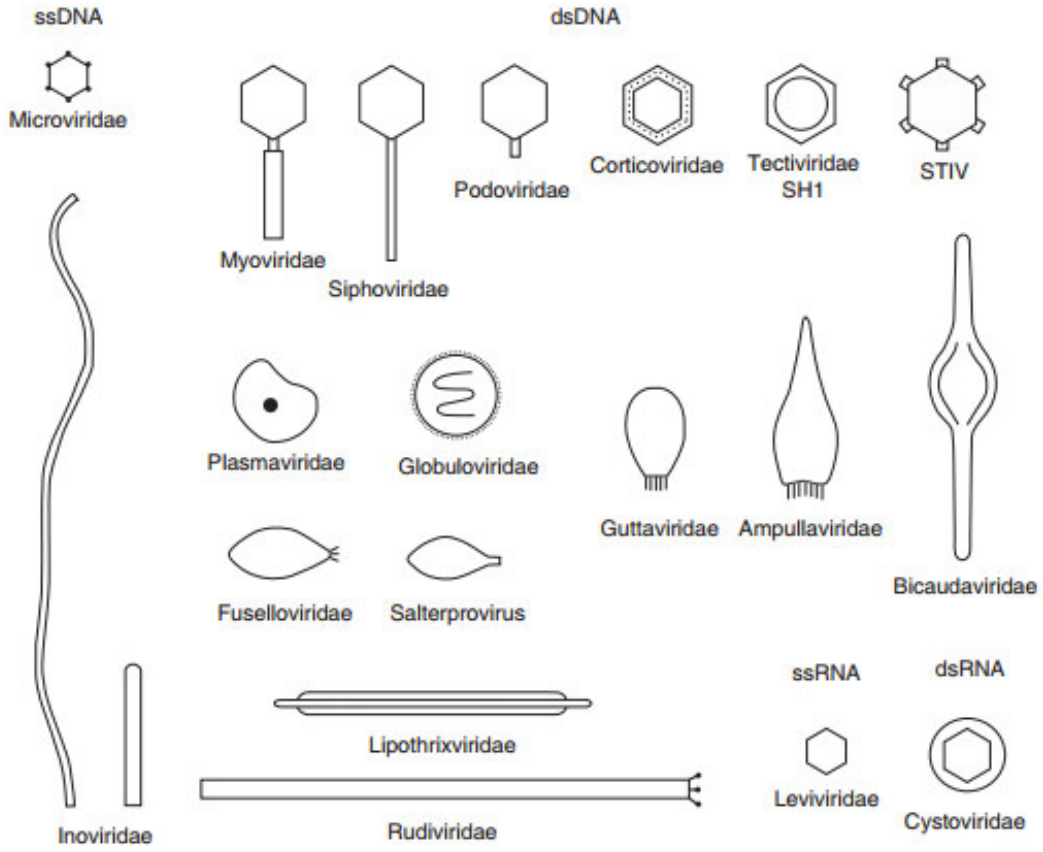

Figure 4- Bacteriophages morphology [72].

The tail consists in a hollow tube through which the DNA passes, in the infection phenomenon, and can be surrounded by a contractile "collar". At the end of the tail, can exist a baseplate and tail fibers bounded to it. Associated to these tail fibers are proteins (tail fiber proteins -TFP) that specifically make the recognition of surface molecules from bacterial cell hosts and that promotes the phage attachment to the bacterial cell hosts [40].

1.3.3 Life cycle

In all life cycles of tailed phages, the infection starts with the phage binding to specific receptor molecules that are exposed in the bacterial host surface, like lipopolysaccharides, proteins, sugars, teichoic acids, lipoproteins, fimbriae, and pili. Usually, this initial attachment is mediated by tail fibers or tail spikes that makes the specific recognition of the exposed bacterial receptors and thus the phage only infects the bacteria with specific receptors, which determines the host range organisms of the phage. According to its host range, the phages can be divided into monovalent phages and polyvalent phages as referred above, being the first ones able to attach to specific species or strains and the second ones are able to adsorb across bacterial species or genera [71,72].

The absorption phase depends on bacteria and phage surrounding environment, like the presence of some cofactors like Mg^{2+} and Ca^{2+} [70]. In some cases, after the adsorption stage the phages can destroy host capsules by the release of enzymes like hydrolases and polysaccharide lyases. The genetic material is then injected by the phage after the induction of pores in bacterial cell wall while the capsid (in most of the cases) remains outside of the bacteria. In the injection step, the phage structure suffers conformational changes that are necessary for tail sheath contraction, which in turn forces the hollow inner tube into the cell allowing the genetic material passing to the cell cytoplasm [70].

The adsorption step is the beginning of bacterial infection, independently of the phage life cycle, that can be lytic or lysogenic (Figure 5) [15,71]. In the lytic cycle (virulent), after the injection of genetic material, the phage genetic material takes over the host cell metabolism, using the host protein machinery to express the viral genes that are responsible for the synthesis of viral proteins and viral nucleic acid replication. Thereafter occurs the packing of DNA, phage assembly and lysis of bacterial host, with the release of new virus particles. The lysis process occurs by the hydrolysis of cell wall from the inside mediated by specific phage encoded enzymes called endolysins [15,40,70,71]. The number of viral particles released depends of the state of bacterial host, the phage itself and of the environmental conditions. The new bacteriophages formed, spread to infect others cells, and normally this cycle occur at maximum in 1h to 2h [70]. The bacterial infection with bacteriophage can be visualized by the appearance of clear plaques on respective bacteria lawns using the double layer agar method [72].

In the lysogenic cycle (temperate phages), the genetic material from viruses is integrated into the genome of the bacterial host and remains in the bacterial chromosome as a prophage, being replicated in conjunction with the bacterial genome and transmitted to bacterial progeny or to other bacteria through transduction, until the induction of lytic cycle by some stimulus (normally associated with adverse conditions). During lysogeny, the production of virions progeny and bacterial lysis is absent [40,70].

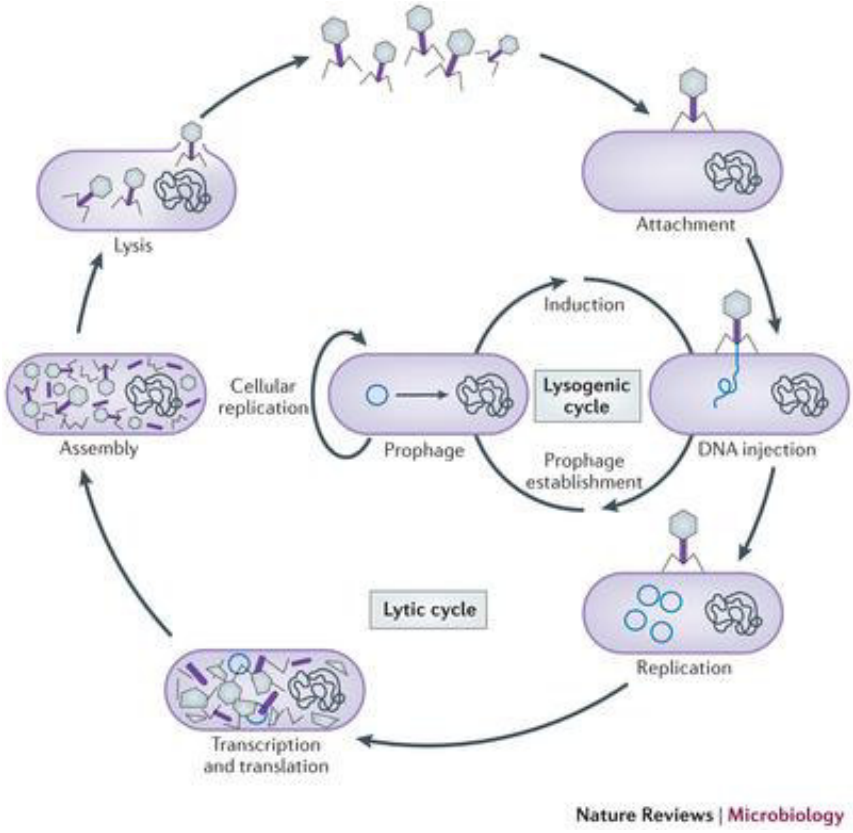

Figure 5- Tailed bacteriophages life cycles and its main steps: the lysogenic and lytic cycles [73].

The insertion of a phage genetic material in the bacteria genome can lead to a phenotype alteration, like the increase of pathogenicity, increase of antibiotic resistance, among others, being the phages involved in the host DNA transduction by a process called by lateral gene transfer [40,70,71].

1.4 Detection using Bacteriophages

Despite the advantages of traditional methods for bacterial detection, the advent of new detection methods has becoming urgent since that the traditional ones suffer of many drawbacks like, the lack of speed, incapability to be applied in the field, high costs, complicated protocols with necessity of specialized operators to perform it, some of them lack sensitivity, and almost all of them need pre-enrichment steps [18,52,74,75].

The emergence of antibiotic-resistant bacteria, due to the large misuse of antibiotics led to a renewed interest on the use of lytic bacteriophages and the advances in phage molecular biology allowed their application as biocontrol agents in food (preventing the food deterioration and the spread of bacterial diseases), in therapy methods for diseases caused by bacterial infections, and as surfaces bio-sanitizers [22,40,41,70]. On the other hand, the use of lysogenic bacteriophages for food industry applications is not recommended since due to their potential in the development of more virulent and resistant pathogens, as referred above [70].

The use of lytic bacteriophages to control pathogens was already successfully demonstrated for *Salmonella enterica*, *E. coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Pseudomonas* reduction in the food [76–80].

Bacteriophages are also an attractive tool for foodborne pathogen detection due to their fast and inexpensive production (taking about 1-2 hours), their predominance on Earth, effectiveness against antibiotic resistant bacteria, they are auto-replicative and auto-limiting into the host cells, they can distinguish viable cells from non-viable cells, infect the bacterial host with high specificity and sensitivity, they influence only a few bacteria from normal flora being harmless to humans and animals, and their stability (they have low susceptibility to pH, temperatures, proteases and organic solvents variations) [21,22,33,37,70,76]. Bacteriophages are thus an excellent choice as probes in the detection field because, as referred before, they make a unique recognition of their bacterial hosts, allowing its application with high specificity [81].

However, bacteriophages have some limitations too: they can carry genes encoding for virulence factors or toxins, the host bacterial can acquire resistance to the phage, with creation of new phenotypes, like the display of different receptors that are no more recognized by the bacteriophages (but some phages can adapt to these bacterial changes) which can alter the bacterial virulence or performance, among others [82,83]. The narrow host range phages may also constitute a disadvantage, and the human

immune system can recognize the bacteriophages as invaders generating an immune response to eliminate them from the circulatory system [22].

The detection methods that use bacteriophages as a tool rely in three steps of the phage lytic life cycle: a) the bacteriophage adsorption to the bacterial surface to form a phage-bacterial complex; b) the labelled viral-genetic material replication into the host; and c) the detection of intracellular components released during the bacterial host lysis. These detection methods can employ different phage-based recognition elements like, wild type phages, reporter phages, and phage-receptor binding proteins, among others [37,43,84].

In traditional methods, the phages can be also applied in the enrichment step. The aim is to use selective phages for microbial competitors that might be present into the sample as a supplement in primary media, to eliminate microbial background and promote the *Salmonella* growth. This concept is the basis of SELECT system (SEDIX) [85]

1.4.1 Detection methods based on phage-bacterial host complex

These methods rely on the high strength of formed phage-bacteria complex due to the high bacteriophage affinity and specificity to the bacterial host receptors (partly mediated by TFP). Thus, phages can be used as bio-recognition elements in biosensors and in other assays to efficiently capture the pathogens. Either bacteriophages or their tail proteins have been included in several types of assays [21,70]. In the case of polyvalent phages, phages that infect different species, false positives may occur [15].

The binding between phage and bacteria can be monitored by phage staining with fluorescence and the complex formation can be visualized through flow cytometry and fluorescent microscopy, with a sensitivities that round the 10^4 CFU/mL and 10^2 to 10^3 CFU/mL respectively [15,68,70]. These values can be improved through the use of fluorescent quantum dots (QDs), which will provide a highest intense and stable signal [15,70,76].

Another approach is the immobilization of bacteriophages and monitor of bacteria binding by optical, electrochemical and microelectromechanical (MEMES) devices in real time (Figure 6) [51,70]. Many studies already used bacteriophages as the biorecognition element in biosensors for bacterial detection, like the surface plasmon resonance (SPR) that is used as a transduction platform in bacteriophage based biosensors reporting detection limits of 10^2 for *E. coli* and 10^4 for *S. aureus*. Magneto-elastic biosensors also apply bacteriophages and have the advantages of being miniaturized and

detect various agents simultaneously. They consist in bacteriophage absorption to the biosensor surface and measure of bacterial cells binding through magnetic resonance, obtaining detection limits around 10^2-10^3 CFU/cm^2 [70,76,86–89]. The limitation of this method relies on the time needed for the analysis and the complex procedure that complicates the sensor preparation [33].

Alternatively, bacteriophages can be attached to paramagnetic beads allowing the capture of bacterial pathogens with higher sensitivity with recovery results compared to antibodies. Phage based biosensors have been successfully used for detection of bacteria directly in fresh products such as milk, broth, fresh tomato, and water [21]. The *Salmonella* detection limits into milk samples with this approach rounds the 1.4-19 CFU/mL with and without a enrichment step respectively, taking 2.5 h to 6h [90].

The application of bacteriophage components instead of the whole phage particle is also a possibility already explored by some companies that use high affinity phage-molecules to attach bacterial host receptors, like TFP and cell wall binding domains (CBDs) from phage endolysins, without the need of an infection step [15,37,70].

The use of TFP or CBDs instead of antibodies or intact phages has numerous advantages: they can be easily modified to improve the bacterial capture, by optimization of its binding properties, combining different TFP or CDBs to obtain improved chimeric proteins; they do not induce cell lysis which is associated with the release of toxic intracellular components and can lead to the loss of signal intensity of biosensor; they present higher stability (temperature, pH and ionic stability); they can overcome the problem of immobilization of large size phages into the biosensors platforms; they do not carry virulence genes and they are not involved in gene transferr, among others [15,35,37,49,51,76]. The main advantages of phage derived particles application as detection tool are listed in Table 1 as well as the bacteriophage advantages.

To detect Gram-negative bacteria, receptor binding proteins (RBPs) present in tail fibers or spikes can be used [15]. For example, the RBPs can be attached to paramagnetic beads to capture bacterial pathogens since they show better sensitivity and percent recovery results compared with antibodies [23,50]. This technology is already applied in VIDAS UP system (Biomerioux), which uses recombinant fluorescent labelled-TFP for *E. coli* O157:H7 detection in food, feed, environmental samples and soil [37–39,70]. Also, using a gold surface based transduction platform with a specific TFP (gp48) attach to nanofuntionalized beads, it was possible the detection of concentrations of 10^2 CFU/mL of *C. jejuni* [51].

Table 1- Main advantages of bacteriophage and bacteriophage-derived particles application as tools in detection methods [40].

Bacteriophages benefits	Added benefits of TFP
Effective against multidrug-resistant bacteria	Do not carry and transfer virulence genes
High specificity- usually have a narrow host range	Do not lyse bacteria avoiding the intracellular toxic components
High diversity	Easier administration and storage
Nontoxic	High stability
Low cost and fast production	Less susceptible to mutations
Already approved by FDA for use in meat and poultry products	Can be expressed in large scale without host pathogen propagation system Recombinant RBP can be improved by genetic engineering to be more specific, or can be fused with others proteins acquiring additional advantages

The TFP have an advantage relatively to the CBDs, that consists in the ability to clear the pathogen by agglutination without lysis and release of bacterial toxic components being this approach demonstrated with chickens infected with *Salmonella enterica* [49,91].

The phage-derived particles have been applied in many methods for foodborne pathogen detection, being the most common the sandwich assays and the biosensors. In the first approach, the cells are first immobilized with antibodies and then the recombinant fluorescent phage proteins are applied to detect the immobilized cells [37]. In biosensors, a recombinant tail spike from *Salmonella* phage P22 attached to a gold surface it was possible to obtain a detection limit of 10^3 CFU/mL in 20 minutes in milk [92] and in 30 minutes a detection limit of 5×10^2 CFU/mL into tomatoes [86]. Although, the use of immobilized phage or phage derived particles in sensors surface has the disadvantage of being difficult to obtain the correct orientation for bacterial capture [70,76] .

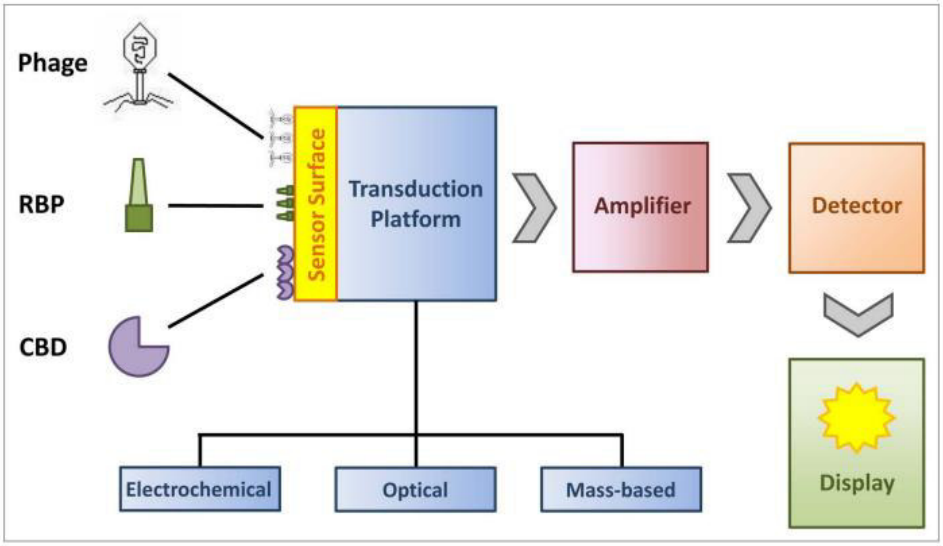

Figure 6- Bacterial pathogens detection through bacteriophage or bacteriophages-derived particles (RBP and CBD) based biosensors. The phages or phage derived components are immobilized to the sensor surface acting as biorecognition elements [37].

1.4.2 Detection methods based on labeled viral-nucleic acids replication

In this approach the genetic material from phages is modified (by direct cloning, transposition, and homologous recombination) to carry out specific reporter genes like luciferase expressing gene (*Lux)*, β-galactosidase (*lacZ)* gene, *celB* , bacterial ice nucleation (*inaW)* gene, yellow fluorescent protein encoding gene (ZsYellow), green fluorescent protein (*gfp)* gene, among others, in order to detect bacterial host during phage replication step or reporter genes expression by bioluminescence, fluorescence, or enzymatic conversion of a chromogenic substrate [15,26,37,49,70].

This method is sensitive, fast, detect only active and viable cells and does not require a previous sample preparation and purification due to the high specificity and robustness of the assay. However, the construction of reporter phage is labor-intensive and requires knowledge about the genetic information of the phage. Additionally, the quantity of inserted information into the phage genome has a natural limit that is set by the phage capsid volume and the application of these assays can be limited since the reported phages are genetically modified organisms (GMOs) that difficult the consumer acceptance and regulatory approval of these assays [15,26,37,70].

The use of reporter phages for bacterial detection has been successfully applied for *Salmonella* [93], *E. coli* [94], *Listeria* [95], and *Mycobacterium* [96].

The *gfp* and *lacZ* genes are small and are not a problem for synthetic phage construction. The *luxCDABE* operon is rarely used, instead, the phage modification normally consist in only *luxAB* and its use requires the exogenous adding of substrate [15].

The most sensitive method is the luminescent assay (LuxAB), once samples do not contain practically background luminescent signal. This is an advantage over fluorescent assays which are affected by the strong auto fluorescence of some samples. The use of *LacZ* as a reporter gene is also limited due to its presence in many bacteria, which can compromises the assay selectivity and lead to false positive results [70].

The reported detection limit for luciferase recombinant phages is 10^{2}-10^{6} cells/mL, however if a pre-enrichment step is used this limit decreases for a few number of cells within one to several hours [37,70]. For *Salmonella* detection a lower detection limit was obtained using *inaW* reporter phage combined with bacterial ice nucleation detection (BIND) assay that allowed the detection of 2 CFU/mL within 3 h in buffer and raw eggs and 10 CFU/mL in samples with high background flora [97]. This technique when combined with IMS is improvs the detection limit to 5 CFU/mL [98].

The detection of bacteriophage amplification can be made without with the use of reporter genes by using phage-specific antibodies that detects phage natural amplification upon target bacterial presence. This technology is applied by MicroPhage and FDA-approved to detect methicillin-resistant *Staphylococcus* [99].

1.4.3 Detection methods based on detectable intracellular bacterial host components

In these assays the lytic phages are used in order to obtain the host cells lysis due to the action of holins, that creates pores in the cytoplasmic membrane, and endolysins that degrades the peptidoglycan. During this stage, it is common the release of intracellular materials besides the phage progeny, which may be used as indicators of this lysis event, and therefore their measure can be used for bacterial pathogen detection [26].

The intracellular ATP release is widely used for bacterial number determination, since this molecule is present in living bacteria in a consistent quantity (10^{-15} g). Using the sensitive bioluminescence assay (luciferase/luciferin enzyme system), phages and ADP, a light emission signal proportional to the ATP amounts obtained allowing the determination of the bacterial cells number present in the sample [15,26,37,70]. However, the background ATP present in food samples leads to an overestimation of the

bacterial loads with corresponding high detection limits (10^4-10^5 CFU/mL). To overcome this limitation, phage immobilized into a filter surface linked to a bioluminescence ATP assay can be used, which concentrates the pathogen and decreases the detection limit for 6×10^3 CFU/mL within 2h with high accuracy [15,91].

Another approach to overcome the high detection limit is the amplification of the bioluminescent signal by measuring the adenylate kinase (AK) activity released during the bacterial cell lysis. The intracellular AK has the function of ADP conversion into ATP, and when is added a high ADP concentration as substrate the AK presence is detected by the high ATP production detected by classical bioluminescent ATP assay. The ADP adding increases the sensitivity of this technique, obtaining detection limits for *E. coli* and *Salmonella* that rounds the 10^3 CFU/mL [100,101]. The detection of *Salmonella* spp., *Listeria* and *E. coli* O157 can already be made by this technique using commercial available protocols that previously applies pathogen capturing by IMS from pre-enrichment media, like for example, FastrAK (Alaska Food Diagnostics) [26,37,39,102].

Besides the AK and ATP phage mediated release measure, bacterial pathogens can be detected through the release of others intracellular components, namely the enzyme β-D-galactosidase, measured by adding its substrate p-aminophenyl-β-D-galactopyranoside, that is converted into p-aminophenol. The p-aminophenol oxidation can be detected by a change in the electric current in a potentiostat device and a sensitivity of 10 CFU/mL can be obtained by previous filtration and re-incubation of the sample prior to phage infection [15,37].

Instead detecting intracellular components released due to bacterial host lysis, the phage amplification assay can be used. This method consists in the bacterial incubation with the phage followed by anti-viral reagent addition before cell lysis in order to remove bacteriophage excess that did not infect the bacteria. Then the anti-viral reagent is neutralized to allow the infection of helper cells by phage progeny. These cells are finally plated onto a soft agar overlay and the result is the appearance of lysis plaques in solid nutrient medium [26]. Alternatively, the cells can be incubated in liquid media and the bacteriophage derived lysis can be observed by the lack of turbidity [41,70].

The phage amplification assays can be combined with antibiotics or other detection techniques (PCR, ELISA) to distinguish susceptible from resistant antibiotic strains in the first case, and additional information in bacterial typing in the second case [26]. The disadvantages of this method rely in the necessity of high initial quantity of bacteria that makes necessary the application of a pre-enrichment and isolation steps resulting in a labor and time intensive method [70]. The reported detection limit for *Salmonella* is 600 CFU/mL in 4h [103].

The presence of a target pathogen can also be monitored by the application of a specific bacteriophage that will lead to bacteria lysis and through assays that measures the metabolic activity and microbial growth by the presence or absence of small high charged intracellular components. The presence of these components are indicative that the bacterial community present in the sample grows and the absence of these components indicates changes in the conductance of bacterial growth by the application of the bacteriophage [70]. The cells lysis derived from phage application leads to changes in the sample conductance showed by a conductance curve. This method has also limitations: cannot be tested in samples with low microorganism's number and can be affected by the food matrix requiring calibration curves for each type of matrix sample. In this assay, the detection of *Salmonella* relies in the measure of large conductance changes associated with the trimethylamine-N-oxide metabolism during *Salmonella* growth [68].

1.5 *Salmonella* detection using recombinant TFP from *Salmonella* phages

The use of TFP as tools for pathogens detection, demands some preliminary studies. Initially, phages infecting the targeting pathogen, in this case *Salmonella*, need to be isolated and selected. Afterwards, the genome of the selected phage must be sequenced and a bioinformatics analysis must be carried to identify potential genes encoding for TFP [104]. The identification of the real TFPs must then be concluded through functional analysis. Ideally, *Salmonella* detection, biocontrol and therapy should make use of a phage able to recognize as many as possible, if not all, *Salmonella* pathogenic strains. Although, the isolation and selection of broad range host phages is a difficult task since their existence in the nature is rare [105]. Some studies have reported the isolation of *Salmonella* phages that successfully detect these bacteria, however they present a narrow spectrum enabling the identification of only some *Salmonella* serotypes as is the case of the RPBs from the well-known P22 phage that specifically infects only *Salmonella enterica* subsp. *enterica* serovar Typhimurium [40].

Santos et al. in the scope of the European project Phagevet-P has characterized a multivalent lytic phage, PVP-SE1, able to infect a broad range of *Salmonella* as well as *E. coli* BL21 and K12. The lytic spectrum of this phage showed, for the *Salmonella* chosen strains, to be even larger than the Felix-O1, a virulent phage well known by its ability to infect a wide range of *Salmonella* [106]. Morphologically, the PVP-SE1 phage belongs to the *Myoviridae* family and due to its genomic and proteomic similarities with rV5 phage it was classified as a"rV5-like virus"[107,108].

Similarly to many *Salmonella* phages, the receptor for PVP-SE1 seems to be the LPS, recognizing also the inner core region of LPS, which may explain the PVP-SE1 broad spectrum, since the LPS inner core is a high conserved region present in many bacteria that belongs to *Enterobacteriaceae* family [108].

According to the phage genome annotation, several genes point for TFP function, as is the case of g*40,* g*41,* g*46,* g*48* and g*51* [108]. However, the TFP responsible for *Salmonella* recognition was not yet identified. The recombinant cloning and expression of several putative tail fibers from PVP-SE1 in fusion with *aceGFP* gene was already tried to understand their ability to recognize and bind to *Salmonella* cells but without positive results. Some reasons might explain the lack of protein binding to the cells: the uncorrect folding of TFP due to the absence of appropriated chaperones that mediates the TFP folding; the need to co-express more than one TFP encoding gene; among others.

Some reports show that in order for the TFP to be functional it needs to be co-expressed with phage chaperones enabling the TFP to acquire the correct tridimensional conformation, solubility or the

formation of trimers. For example, the recombinant short and long TFP from T4 phage, namely gp12 and gp37 proteins, for a correct conformation and posterior *E. coli* binding, require, during its expression, the presence of gp38 and gp57 chaperones [109–113].

The difficulty to identify the PVP-SE1 TFP and the possible chaperones leads to a slight different approach in the TFP identification: co-expression of the PVP-SE1 potential TFP with chaperones from other *Myoviridae* phages for which the chaperones have been identified. A good example is the T4 phage for which the gp57 and gp38 proteins have been identified as a two-chaperone system needed for the T4 TFP to be functional and which can be extensible to the expression of long TFP from others *Myoviridae* phages, according to *Bartual et al.* [113].

Besides the isolation of PVP-SE1, the group has also isolated the PVP-SE2 *Salmonella* phage (former phi38), that belongs to the *Siphoviridae* family, it is a lytic phage and infects a wide variety of *Salmonella* bacteria [106,114] This phage, similarly to PVP-SE1, presents potential in the development of a tool for *Salmonella* detection using its TFP as recognition elements. The genes g*27*, g*28*, g*40*, g*47* and g*54* were identified through the genome annotation as potential TFP. Also, three genes were identified that might encode for PVP-SE2 TFP assembly chaperones: g*32*, g*33* and g*34.*

1.5.1 Objectives

In this work, it is intended to identify, express and characterize TFP able to specifically detect the pathogenic bacteria *Salmonella*. The genes encoding for TFP were identified *in silico* from two *Salmonella* infecting phages' genomes (PVP-SE1 and PVP-SE2). PVP-SE1 is a broad lytic spectrum phage and therefore its TFP can provide an excellent tool for the detection of most of the *Salmonella* strains commonly isolated from contaminated food. Due to the potential need for chaperones co-expression and the difficulty to identify them, the well characterized phage T4 will be used as a model and its chaperones co-expressed with the potential TFP of phage PVP-SE1. Functional analysis of the phages TFP were carried through their cloning in fusion with a *aceGFP* gene and heterologous expression in *E. coli* BL21(DE3). The constructed chimera, when composed by a functional TFP, enables the visualization of the natural phage host *Salmonella* cells under the fluorescent microscope.

After identification of functional TFPs, they were used to assess their binding ability against different *Salmonella* strains/serotypes to understand the potential of these TFPs in the development of a detection tool.

Chapter 2

Methods and proceedings

2.1. Bacterial strains, plasmids and phage DNA

The bacterial strains used to clone and heterologous express the encoding TFP and chaperones genes were *E. coli*, due to the facility to be manipulated and the high pool of tools available for this organism. Namely, the ones used for molecular cloning were *E. coli* TOP10 chemically or electro-competent cells (New England Biolabs). The *E. coli* BL21 (DE3) (New England Biolabs) chemically competent cells (prepared as described in annex I) were used to express the selected proteins.

The bacterial strains used to assess the binding ability of the expressed TFP were *E. coli* BL21 (DE3) and a variety of *Salmonella* strains represented in Table 16 (section 3.3), which includes the PVP-SE1 and PVP-SE2 host phage strains [106]. Bacterial growth was always performed on Lysogeny Broth (LB Broth - Liofilchem) at 37 °C and 120 rpm.

Salmonella enterica serovar Typhimurium LT2 and its derived LPS mutants (Table 17), rough and deep rough mutants, were obtained from the *Salmonella* Genetic Stock Centre (University of Calgary, Alberta, Canada) [115]. These cells were used to test the ability of phages to adsorb and TFP to bind, to different portions of LPS [108].

The used bacteria and the clones containing the correct constructs were stored at -20 °C in LB broth supplemented with 20% (v/v) glycerol (Fisher Bioreagents).

The commercial plasmids pET15b, pETduet and pCDFduet (Novagen) (Restriction map and main characteristics available in annex II) were used to clone the chaperones. The pGFP plasmid already existed in the group and consists in a construction of the commercial plasmid pET28a (Novagen) with the synthetic construct aceGFP (GenBank: AY233272.1) inserted in the multiple cloning site (MCS) between the *NdeI and BamHI* restriction enzymes sites. This plasmid was used for the in-frame cloning of TFPs at the C-terminus with the aceGFP at the N-terminus.

The recombinant plasmids p40_se1, p41_se1 and p51_se1 consist in the insertion of the putative TFP encoding genes g*40,* g*41* and g*51*, from PVP-SE1 phage in the pGFP plasmid and were constructed and available in the group. These plasmids were used to co-express with T4 and PVP-SE2 chaperones to assess their binding ability to *Salmonella* and *E. coli* bacteria.

PVP-SE2 and T4 (ATCC® 11303-B4, sequence available at NCBI database: NC_000866.4) phages DNA were obtained through its isolation from the phage lysates using the kit NucleoSpin® Virus (Macherey Nagel Bioanalysis), according to the manufacturer's instructions.
Both PVP-SE1 and PVP-SE2 phages were isolated and characterized in the research group [106,108].

2.2. Cloning of tail fibers proteins and chaperones from PVP-SE2 and T4 phages

Five genes from the *Salmonella* phage PVP-SE2, namely g*27, 28, 40, 47,* and *54* (nucleotide and amino acidic sequence available in annex III), that according to phage genome annotation are putative tail fibers proteins encoding genes were selected for subsequent cloning individually in pGFP plasmid (in frame with aceGFP gene) for posterior expression. The same approach was used to clone the tail fiber proteins encoding genes from T4 phage, namely g*37* and g*12* (nucleotide and amino acidic sequence available in annex III), as a control and validation method to detect *E. coli* strains. C-terminal truncated sequences of the T4 *gene 37* (g*37@726* and g*37_1380*) which might contain the domain of g*37* responsible for the T4 phage attachment to *E. coli* bacteria were also cloned into the pGFP plasmid.

The cloning of the tail assembly chaperones-encoding genes, for both PVP-SE2 and T4 TFPs, was performed using the commercial plasmids pET15b, pETduet and pCDFduet. The T4 chaperones encoding genes that have already been identified as g*57* and g*38* [109–113,116–122] (nucleotide and amino acidic sequence available in annex III) were cloned into the MCS 1 and MCS 2 of pCDFduet or pETduet plasmid, respectively. For PVP-SE2 phage chaperones cloning a bioinformatics analysis was performed and identified g*34*, g*33* and g*32* (nucleotide and amino acidic sequence available in annex III) as potential TFP chaperones. Given the size of the three genes together and the lack of knowledge on the need of all these putative chaperones they were intended to be cloned all together but also in two separated inserts/sequences (g*34-32* and g*34-33*+g*32*) in the same or separated plasmids.

2.2.1. Primers design

The primers used to amplify the TFPs and chaperones encoding genes are presented in Table 2 and Table 3. These were designed considered the restriction sequence (highlighted nucleotides), the annealing sequence and when necessary the required nucleotides to maintain the reading frame (underlined). The online tool OligoAnalyzer 3.1 (available at: https://eu.idtdna.com/calc/analyzer) was used to assess some parameters as the melting temperature (Tm), the GC content, primers homo and self-dimers and primers hairpin.

Table 2- Primers used to amplify the TFP and chaperones encoding genes from PVP-SE2 and their parameters. The nucleotides highlighted in bold correspond to the restriction enzyme sequences.

Gene	Sequence (5'→ 3')	Enzyme	NN	Tm	GC content
27	Fw: GCCGCC**GAGCTC** ATG TCC AGC GGT TGC GGT G	*SacI*	19	61.2	63.2
	Rv: CCGCCG**CTCGAG** TTA TGC CAA AGT TAA TCT TGT ATA GCT TCC	*XhoI*	30	55.5	33.33
28	Fw: CCGCCGCATATG**GAGCTC** TTG GCG CTA GTA ATC CAC TAT ACC	*SacI*	24	56.2	45.8
	Rv: CCGCCG**CTCGAG** TTA GTT AAA ACC GTT ATC GAA TCC GCT	*XhoI*	27	56.5	37
32 fused with *34-33*	Fw: CCGCCG**GGATCC** ATG GCT GAT GTA GCT AGC TTA GTA G	*BamHI*	25	55.8	44
	Rv: CCGCCG**CATATG** TCA ATA TCC TGG TTG GCC GCG	*NdeI*	21	60.1	57.1
32	Fw: CCGCCG**CCATGG** ATG GCT GAT GTA GCT AGC TTA GTA G	*NcoI*	25	55.8	44
	Rv: CCGCCG**CATATG** TCA ATA TCC TGG TTG GCC GCG	*NdeI*	21	60.1	57.1
34-33	Fw: CCGCCG**CCATGG** GTG GTT CCC GCC TGG CG	*NcoI*	17	62.7	76.5
	Rv: CCGCCG**GGATCC** TCA GCC ATC GTT TCT ACC CTC AAA AAT G	*BamHI*	28	59.1	42.9
34-32	Fw: CCGCCG**CCATGG** GTG GTT CCC GCC TGG CG	*NcoI*	17	62.7	76.5
	Rv: CCGCCG**CATATG** TCA ATA TCC TGG TTG GCC GCG	*NdeI*	21	60.1	57.1
40	Fw: CCGCCGCATATG**GAGCTC** ATG GCG TTA CAA CCA TAT AAG GGC	*SacI*	24	57.4	45.8
	Rv: CCGCCG**CTCGAG** TTA GGT GTA CTT AAT GCG CTG GAT AA	*XhoI*	26	56	38.5
47	Fw: GCCGCC**GAGCTC** ATG GTT GAT GTA ATT AAA CGT CGT ATT GTT	*SacI*	30	55.7	30
	Rv: CCGCCG**CTCGAG** TTA ACT TAC GGT TAC TAC TGT GCT ATC	*XhoI*	27	54.2	37
54	Fw: GCCGCC**GAGCTC** ATG GGC TTT TTC AAA GTT AAA GAT GTG C	*SacI*	28	56.9	35.7
	Rv: CCGCCG**CTCGAG** TTA CTT AGG TTC TGA GAC CTT AGC	*XhoI*	24	57.3	41.7

Table 3- Primers used to amplify the TFP and chaperones encoding genes from T4 phage and their parameters. The nucleotides highlighted in bold are the restriction enzyme sequence and underlined are the ones necessary to keep the reading frame.

Gene	Sequence (5'→ 3')	Enzyme	NN	Tm	GC content
12	Fw: GCCGCC**GAGCTC** ATG AGT AAT AAT ACA TAT CAA CAC GTT TCT AAT G	*SacI*	34	54.2	26.5
	Rv: CCGCCG**CTCGAG** TCA TTC TTT TAC CTT AAT TAT GTA GTT TAA AGA AAT G	*XhoI*	37	54	21.6
37	Fw: GCCGCC**GTCGAC**AA ATG GCT ACT TTA AAA CAA ATA CAA TTT AAA AGA AG	*SalI*	35	54.5	22.9
	Rv: CCGCCG**CTCGAG** TTA TGC TAA ACG AAC GAT ATA GTT AAA AGC A	*XhoI*	31	55.3	29
37@726	Fw: GCCGCC**GAGCTC** GGT AAT ATC ACT GGT GGT TCT GG	*SacI*	23	55	47.8
	Rv: CCGCCG**CTCGAG** TTA TGC TAA ACG AAC GAT ATA GTT AAA AGC A	*XhoI*	31	55.3	29
37_1380	Fw: GCCGCC**GAGCTC** GAT GG CAC AAG GAC TAT CCA ATG G	*SacI*	24	57.7	50
	Rv: CCGCCG**CTCGAG** TTA TGC TAA ACG AAC GAT ATA GTT AAA AGC A	*XhoI*	31	55.3	29
38	Fw: CCGCCG**CATATG** ATG AAA ATA TAT CAT TAT TAT TTT GAC ACT AAA GAA T	*NdeI*	37	52.5	16.2
	Rv: CCGCCG**CTCGAG** TTA TAC GTA ATG CTT GAA TAA ACG CTT ATA	*XhoI*	30	53.4	26.7
57	Fw: CCGCCG**CCATGG**_GC ATG TCT GAA CAA ACT TGT TGA ACA AAA AC	*NcoI*	29	55.6	31
	Rv: GCCGCC**GAGCTC** TCA TTC TTC ATC TTT TGC TTC ATC TG	*SacI*	26	53.9	34.6

2.2.2. Gene Amplification

The target genes were amplified using the phage DNA (T4 or PVP-SE2) as template and the *Phusion Green High-Fidelity DNA polymerase* (Thermo Scientific). The conditions to amplify the genes were set according to the manufacturer instructions and are presented in Table 4 and Table 5. The PCR amplification was conducted in a MJ Mini Gradient Thermal Cycler (BioRad).

Table 4- PCR mix components and their final concentrations to amplify the TFPs and chaperones genes.

Mix components	Final concentration
Ultrapure Water	up to the desired volume
5X Phusion Green HF Buffer	1X
dNTP mix (10 mM)	200 µM
Primers	0.5 µM
Phage DNA template	0.9 ng/µL
High- fidelity Phusion polymerase	0.02 U/µL

Table 5- PCR protocol used to amplify the TFPs and chaperones genes.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	35
Annealing	65	30 s	35
Extension	72	30 s - 2 min min *	35
Final extension	72	10 min	1
Hold	4	Forever	-

*It was used 30 s min per 1 Kbp as advised.

After the amplification, the correct PCR products were confirmed by their size in a 1% agarose (GeneOn) gel prepared in 1X TAE buffer (1 mM Ethylenediamine tetraacetic acid- EDTA; 40 mM Tris base; 20 mM acetic acid) stained with GreenSafe Premium (Nzytech) and using as ladder the 1 Kb DNA ladder

(New England BioLabs). The gels were run at 100 V for 40 minutes in a Mini Gel II Complete Horizontal Electrophoresis System (VWR) and the visualization of correct bands was done using a ChemiDoc™ XRS+ System with Image Lab™ Software (Version 5.1 Bio-Rad Laboratories Inc.).

Thereafter, the PCR products were purified using the Kit Nucleospin® PCR Clean up (Macherey Nagel). For PCR products with unspecific bands, they were run in a 1%, agarose gel and the correct band was excised from the gel and cleaned using the Kit Nucleospin® PCR Clean up.

2.2.3. Digestion and Ligation

The amplified and purified genes were double digested with *FastDigest™* enzymes (Thermo Scientific) as presented in (Table 6). Digestions were performed in a final volume of 20 µL at 37°C for 1-4h and inactivated at 80 °C during 15 minutes as suggested by the manufacture instructions (Table 7). The complete plasmids digestion it was confirmed by 0.8 % agarose gels being used 6× NZYDNA loading dye (Nzytech).

Table 6- Restriction enzymes combinations used for each gene cloning approach.

Phage origin	TFP		Chaperones	
	Genes	**Enzymes**	**Genes**	**Enzymes**
T4	*12* *37* *37@726* *37_1380*	*SacI + XhoI*	*38*	*NdeI + XhoI*
			57	*NcoI + SacI*
PVP-SE2	*27* *28* *40* *47* *54*	*SacI + XhoI*	*34-32*	*NcoI + NdeI*
			32	*NcoI + BamHI*
			34-33	*BamHI + NdeI*

Table 7- Components and quantities used for inserts or plasmids double digestions.

Mix components		Final concentration
Ultrapure Water		Until 20 μL
Fd buffer		2 μL
DNA	plasmid	1500 ng
	insert	1000 ng
Enzyme 1		1 μL
Enzyme 2		1 μL

To prevent recircularization of the digested plasmids the phosphates groups present in their sticky ends were removed by adding 1 μL of OPTIZYME™ Alkaline Phosphatase and 2.3 μL Alkaline Phosphatase buffer (Thermo Scientific). The reaction was incubated at 37 °C for 1h and inactivated at 75 °C during 5 minutes.

The T4 DNA Ligase from Thermo Scientific or New England Biolabs was used to catalyze the ligation between digested plasmids and digested inserts in a final volume of 20 μL according to the manufacturer's (Table 8) instructions. The reactions were incubated at 22 °C and 16 °C, respectively for 3h to overnight for both enzymes, and inactivated both at 65 °C for 10 minutes.

Table 8- Components and quantities used for inserts and plasmids ligation.

Mix components	Final concentration
Ultrapure Water	Until 20 μL
T4 DNA ligase buffer	2 μL
Insert: plasmid molar ratio	5:1 (1) 3:1 (2)
T4 DNA ligase	0.5 μL (1) 1 μL (2)

(1)- for T4 DNA ligase from Thermo Scientific; (2)- for T4 DNA ligase from NEB.

Primers design, gene amplifications, digestions and ligations were simulated *in silico* using SnapGene™ 1.1.3 version Software.

2.2.4. Transformation

The ligation reactions were used to transform chemically or electro-competent cryopreserved *E.coli* by heat shock during 60s at 42 °C (performed similarly to addgene web page recommendations [123]) or by electroporation (transformation protocols described in annex IV) respectively. After a 2h incubation at 37 °C and 120 rpm in super optimal broth with catabolite repression (SOC). The suspensions were spread in LB agar (20 g/L of LB, 12 g/L of agar- Liofilchem) petri dishes with the respective antibiotic(s) (100 μg/mL of spectinomycin for pCDFduet, 50 μg/mL of kanamycin for pGFP, and 100 μg/mL of ampicillin for pETduet and pET15b) to select the cells that incorporated the recombinant vectors. The plates were finally incubated overnight at 37 °C.

2.2.5. Colony Screening and confirmation

The colonies grown because of the transformation were then screened by colony PCR for the incorporation of the correct construction. To accomplish that, various colonies were randomly picked and grown in 50 μL of LB medium with correct antibiotic for about 1h to be used as template in the PCR reaction. The PCR reaction was carried using the *DreamTaq™* DNA polymerase (Thermo Scientific) and the reaction set according to the manufacurer's recommendations as can be visualized in Table 9. The primers combination used for each clone confirmation are represented in Table 10. In the case of g*37* and g*28* the primers used were the same as for the gene amplification.

Table 9- PCR reaction mixture for colony PCR using *DreamTaq™* DNA polymerase.

Mix components	Final concentration*
Ultrapure Water	Until complete the volume
10X DreamTaq Green Buffer	1.5 X
dNTP mix (10 mM)	1 mM
Primers (10μM)	0.5 μM
High- fidelity Phusion polymerase	0.04 U/μL
DNA template	2 μL of bacterial suspension

The PCR colony products obtained were visualized in 1% agarose gels, and the suspensions from colonies presenting bands with the correct size were spread onto LB Agar plates with the corresponding

antibiotic and incubated overnight at 37°C. The plasmids were then extracted with the kit NucleoSpin® Plasmid (Macherey-Nagel) and the concentration quantified in a NanoDrop 1000™ (Thermo Scientific) spectrophotometer. The correct insertion of the genes was confirmed through sequencing with the T7 forward or reverse universal primers (or the seqGFP for the putative TFPs)

Table 10- Primers used for colony PCR.

Primer	Genes	Sequence (5'→ 3')
SeqGFP forward	TFPs	TGATCTACTTCGGCTTCGTG
T7 reverse	TFPs	GCTAGTTATTGCTCAGCGG
T7+Forward	*34-32, 32, 38*	GATCCCGCGAAATTAATACGACTCACTATA GGG
T7+ Reverse	*34-32,32, 38*	CAAGGGGTTATGCTAGTTATTGCTCAGCGG
F90	*34-33, 57*	CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGG
R76	*34-33, 57*	GAAATTGTTATCCGCTCACAAT

The *E. coli* Top10 cells harboring the correct recombinant plasmids were cryopreserved in LB with 20% glycerol and the corresponding antibiotic and stored at -20°C.

2.2.6. Cloning into expression cells

The correct plasmid DNA confirmed by sequencing was extracted with the kit NucleoSpin® Plasmid (Macherey-Nagel) and 50-100 ng were used for transformation *E. coli* BL21(DE3) cells (as in section 2.2.4) for posterior protein expression and the colonies confirmed as described in section 2.2.5. The cells transformed with chaperones p57-38, p34-33, p32, and p34-33 + p32 were used to make chemically competent cells as described in annex I and further with all recombinant plasmids containing the T4, PVP-SE2 TFP, and PVP-SE1 TFP.

2.3. Other cloning approaches

The normal cloning approach described above was performed for all the tail and chaperone encoding genes however, because gp*28* and gp*34-32* from PVP-SE2 have a high size, namely 2559 bp and 3169 bp, and after several attempts to clone them without any positive results, a different cloning approach for these two genes was tested. The both methodologies used were the Circular Polymerase Extension Cloning (CPEC) and Gibson Assembly high efficient cloning techniques [124,125]

2.3.1. Gene amplification

For these new approaches, new primers were designed (Table 11), considering that the same plasmids were intended to be use in these strategies (pGFP plasmid for g*28* and pCDFduet for g*34-32*). The need for new primers happens due to the required, in these strategies, inserts with ends presenting homology with the cloning vector.

Table 11- Primers used to amplify g*28* and g*34-32* with overlapped regions in their extremities. The nucleotides underlined are the regions that overlap with the correspondent plasmid and the remaining nucleotides are the ones responsible for de annealing to the DNA template. The parameters of these primers were obtaining considering only the overlap sequence.

Primers	Sequence (5'→ 3')	NN	Tm	GC content
28_CPEC	Fw: GGATGAGCTGTACAAGGGATCCGAATTCGAGCTC TTG GCG CTA TGA ATC CAC TAT AC	34	64.9	52.9
	Rv: TGCGGCCGCAAGCTTGTCGACGGAGCTC TTA GTT AAA ACC GTT ATC GAA TCC GC	28	71	67.9
34-32_CPEC	Fw: CAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAA GGAGATATACCATGGGC GTG GTT CCC GCC TGG CG	39	55.9	23.1
	Rv: GATGATGGTGATGGCTGCTGCCCATGG TCA ATA TCC TGG TTG GCC GCG	27	65.3	59.3

With the designed primers, these two inserts were amplified and purified as described before in the gene amplification section (2.2.2) but using as annealing temperature 55 °C. After obtaining the inserts, the plasmids were prepared by linearization with *Fast digest* restriction enzymes that cuts in the place pretended to clone the inserts (*SacI* for pGFP and *NcoI* for pCDFduet) (plasmids linearization performed according to Table 12).

Table 12- Reaction components for pGFP and pETduet linearization**.**

Mix components	Final concentration
Ultrapure Water	Until 20 µL
Fd buffer	2 µL
Plasmid (pGFP or pETduet)	1500 ng
Restriction enzyme (*SacI* or *NcoI*)	1 µL

2.3.2. Circular Polymerase Extension Cloning (CPEC)

The CPEC was performed according to *Quan and Tian* (2011) in a 20 µL final volume containing non-linearized or linearized plasmid and insert (Table 13). The PCR running conditions was set up as described in Table 14.

Table 13- CPEC reaction components and quantities conditions**.**

Mix components	Final concentration
Ultrapure Water	up to the desired volume
5X Phusion Green HF Buffer	4 µL
dNTP mix (10 mM)	0.4 µL
Insert	200 ng
Plasmid	100 ng
DMSO	06 µL
High- fidelity Phusion polymerase	0.2 µL

The resulting recombinant plasmids were confirmed by loading 4 µL of the CPEC reaction in a 1 % agarose gels. The correct sizes should be ≈ 8 kbp for gp*28* insertion in pGFP and ≈ 6 kbp for gp*34-32* in pCDFduet. From each CPEC reaction, 5 µL were digested with *DpnI* enzyme (Nzytech) to degrade methylated DNA to minimize the background.

Table 14- CPEC reaction running conditions**.**

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	1min	1
Denaturation	98	10 s	30
Slow ramp Anneal	70	1 ºC /seg	30
Annealing	55	30 s	30
Extension	72	4 min *	30
Final extension	72	10 min	1
Hold	4	forever	-

*recommended at least 20 s per kb for final cloning product with a full length higher than 4 kb. It this case it was used 30s.

The digested (5 μL) and non-digested (10 μL) CPEC reactions with *DpnI* were used to transform *E. coli* Top10 competent cells and the screening and confirmation of positive colonies was carried as referred in section 2.2.5.

2.3.3. Gibson Assembly

The Gibson Assembly technique was performed only for gp*34-32* cloning using the kit (New England Biolabs) according to the manufacturer's instructions. Briefly, 0.2 pmol of pCDFduet linearized with *NcoI* and 0.2 pmol of gp*34-32* amplified with gp34-32_CPEC primers were added to 10 μL of the reaction master mix and incubated at 50 °C by 1 hour. Afterwards, 5 μL were used to transform *E. coli* Top10 competent cells, and the screening of positive colonies was done was described before (section 2.2.5).

2.4. TFP Expression and Co-expression with chaperones

The expression cells harboring the desired proteins to be expressed were grown overnight in 5 mL LB medium supplemented with the appropriate antibiotic (100 μg/mL of spectinomycin for cells with p57-38 and p34-33, 100 μg/mL of ampicillin for cells with p32, 50 μg/mL of kanamycin for cells with only TFPs, and 50 μg/mL of kanamycin plus the respective antibiotic for chaperone plasmid combination for cells with TFP and chaperones) at 37 °C and 120 rpm in an Orbital Shaker–Incubator ES-20/60 (Biosan).

In the next day 500 μL of pre-inoculums were added to 50 mL of LB media containing the appropriate antibiotic(s) and incubated at 37°C (120 rpm). At an OD (620 nm) = 0.5-0.6 protein expression was induced by adding Isopropyl β-D-1- thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The expression was carried out 8h to overnight at 30 °C and 120 rpm in a MIR-254-PE Cooled Incubator (Panasonic).

2.5. Bacterial disruption and protein analysis through SDS-PAGE

The endogenous production of the recombinant proteins requires a lysis step to recover the soluble recombinant proteins for posterior confirmation of its correct expression by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent purification. For that, the bacterial cultures after overnight expression were harvested and pelleted (15 min, at 4 °C and 9000×g using a 3-16k centrifuge from Sigma-Aldrich (St. Louis, MO)), and then ressuspended in 5 mL of Lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, at pH 7.2). The cells were first subjected to 3 cycles of freezing at -80 °C and thawing at 30 °C in an Unstirred Water Bath (Clifton Range), and thereafter lysed by a Vibra-Cell™ VC505 sonicator (Sonics & Materials, INC) during 5 min (30 sec pulses ON, 30 sec pulses OFF, 40% amplitude intensity) on ice to prevent proteins denaturation. The suspension was centrifuged 15 min, at 4 °C and 9000xg and. the supernatant recovered and filtrated using a 0.2 μm polyethersulfone (PES) membrane filter (Whatman) and then stored at 4 °C.

Samples of 100 μL for subsequent protein content analysis by SDS-PAGE were taken before sonication (total protein) and after, from the supernatant (soluble fraction) and from the pellet ressuspended in a water volume equal to the supernatant (insoluble fraction). Samples were prepared for analysis by adding 10 μL of the sample to 10 μL of Blue Loading Buffer pack (New England Biolabs) and boiling the mixture at 95 °C for 5 minutes. Then the prepared samples were load onto the wells of a 12% polyacrylamide gel (prepared as described in annex V), as well as 3.5 μL of EZ-Run™ Pre-Strained *Rec* Protein Ladder (Fisher Bioreagents). The electrophoresis was carried out in 1×Trisglycine-SDS buffer (TGS) and the power supply was automated for 20 min at 80V and 1h40 min at 120V. The gels were stained using BlueSafe protein stain (nzytech) for 30 minutes and the revealed bands were analyzed. The TFP with cell binding ability were also subjected to electrophoresis in their native form in 8% polyacrylamide gels (without SDS) in non-denaturant conditions using 1× Tris-glycine (TG) buffer, at 4 °C. and were run slowly at 80 V without time control, using as indicative the dye front migration. The samples were prepared in loading sample buffer (without SDS and β-mercaptoetanol) and without heating.

2.6. Recombinant protein purification, concentration and quantification

The filtrates containing the target recombinant TFP were purified using HisPur™ Ni-NTA Resin (Thermo Scientific) with affinity to the histidine tag (6× Hist) present in the N-terminal of the recombinant proteins using Gravity-flow Columns according to the manufacturer's instructions. Briefly, the columns were prepared by packing 500 μL of Ni-NTA Resin, equilibrated two times with 1 mL of Equilibration Buffer (Lysis buffer with 10 mM imidazole). The protein filtrate was loaded into the column and the flow through collected. The column was washed twice with 5 mL of wash buffer (lysis buffer with 30 mM imidazole) to remove non-target proteins. Finally, the recombinant protein was eluted in two fractions with 2x 750 μL of lysis buffer with 100 mM imidazole and 2x 750 μL of lysis buffer with 300 mM imidazole and stored at 4 °C.

The purified protein was concentrated and dialyzed against 10 mM Tris-HCl pH7.1 using Amicon® Ultra 10 KDa 0.5 mL Centrifugal Filters (Merck Millipore) per the manufacturer's recommendations. Briefly, up to 500 μL of the purified protein was loaded in the column and centrifuged at 14 000×g for 20 minutes, 4 °C, repeating this step until all purified protein was loaded. The flow-through was discarded and the column washed twice with 500 μL of 10 mM Tris-HCl (14 000×g, 20

minutes, 4 °C). The concentrated and dialyzed protein was recovered through the addition of 100 μL of 10 mM Tris-HCl turning the column upside down, centrifuged at 1 000×g for 5 minutes.

The concentrated protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, dilutions of the concentrated protein in 10 mM Tris-HCl were made from which 25 μL were taken and added to 200 μL of WR solution (50:1, BCA Reagent A: BCA Reagent B) in duplicate. The mixture was incubated at 37°C for 30 minutes and cooled to room temperature measuring OD at 562 nm. The concentration was calculated by interpolation using a Bovine serum albumin (BSA) calibration curve (OD at 562 nm vs BSA concentration in mg/mL, annex VI) and the dilution factor.

2.7. Fluorescent microscope assays

The filtrates of each TFP expression obtained after the cells disruption were used to verify the ability to bind to *E. coli* and *Salmonella* cells. For that, a single colony from each bacterial strain to be tested was grown in 1 mL of LB at 37°C, 120 rpm, for about 2h. Then the cells were pelleted (8000xg, 3 minutes) and washed with 1 mL 0.9% NaCl twice and ressuspended in 100 μL of the same solution.

Binding to the cells were carried through the addition of 10 μL of the cells suspension to 20 - 200 μL of TFP filtrate (depending on the protein concentration) and incubated for 30 minutes at room temperature. The unbounded TFPs were washed three times with 1mL 0.9% NaCl, 5000xg, 5min. Cells were then ressuspended in 50 μL and 10 μL were mounted in a microscope slide and observed in an Olympus BX51 fluorescent microscope.

The TFP able to bind to the phage host were then tested with different *Salmonella* strains, including all *Salmonella* subspecies and different LPS *Salmonella* mutants. To determine the minimum TFP concentration able to decorate and identify *Salmonella* cells at the fluorescent microscope different TFPs concentrations were used to incubate with the cells: 63 μM, 20 μM, 10 μM, 5 μM, and 2 μM.

2.8. PVP-SE2 phage lytic spectrum

To compare the lytic spectrum of phage PVP-SE2 against the different *Salmonella* strains and the binding ability of the functional TFP heterologous expressed the lytic spectrum of the phage was determined through the spot test using the double layer agar method [108]. For that, bacterial lawns of

the *Salmonella* strains to be tested were prepared by adding 100 μL of bacteria (grown in LB medium overnight at 37 ᵃC, 120 rpm) to 5 mL of top agar (0.5% agar) to prepared LB agar plates and let dry. Then, 5 μL drops of phage suspension were added to the bacterial lawns and incubated overnight at 37 °C. *Salmonella* strains for which an inhibition halo was observed were then tested with serial dilutions of (10^{-1} to 10^{-7}) of PVP-SE2 phage (with an initial concentration of 10^{9} PFU/mL) in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl at pH 7.5), to verify the formation of individual phage plaque to distinguish lysis from without from phage infection.

Chapter 3

Results and Discussion

3.1. TFP and chaperones cloning

Based on the phage genome sequence and annotation previously made in the group, proteins presenting homology with TFP and chaperones were selected. These sequences were successfully amplified by PCR as described in section 2.2.2 section. In the case of genes *27*, *28* and *34-32*, besides the desired PCR product, some unspecific products were obtained even after optimization of the annealing temperature, as can be visualized in Figure 7. The appearance of these unspecific bands may be a result of non-optimal concentration of Mg^{2+} in the PCR reaction mix, formation of primer dimers or the presence of additional priming sites, among others like the equipment failure [126]. Therefore, to obtain the correct inserts, these PCR products were separated into a 0.8 % agarose gel and the correct band was excised and purified, avoiding this way the presence of non-target inserts in posterior cloning steps that would limit the insertion of the target gene and consequently lead to incorrect recombinant plasmids.

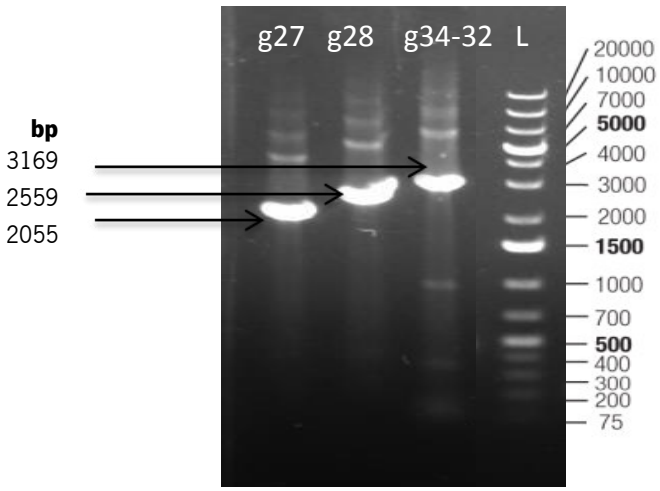

Figure 7- Example of a 1% Agarose gel containing the PCR product for g*27*, g*28* and g*34-32*, which includes the bands with the correct sizes (2055 bp, 2559 bp and 3169 bp) for each gene as well the unspecific bands. The correct bands are marked with the arrows. L = Generuler 1Kbp Plus DNA Ladder (ThermoFisher).

The amplified and purified TFP and chaperones genes were then digested and ligated to the correspondent digested plasmids, as described in section 2.2.3. and used to transform *E. coli* TOP 10 competent cells. Resulting colonies were randomly selected and subjected to colony PCR with the T7 universal primers. The product of each colony PCR was visualized on a 1% agarose gel as can be seen in Figure 8 and Figure 9. In these figures, it can be observed a positive band for each TFP and chaperone cloning, as well as the negative bands that are obtained for the empty plasmids.

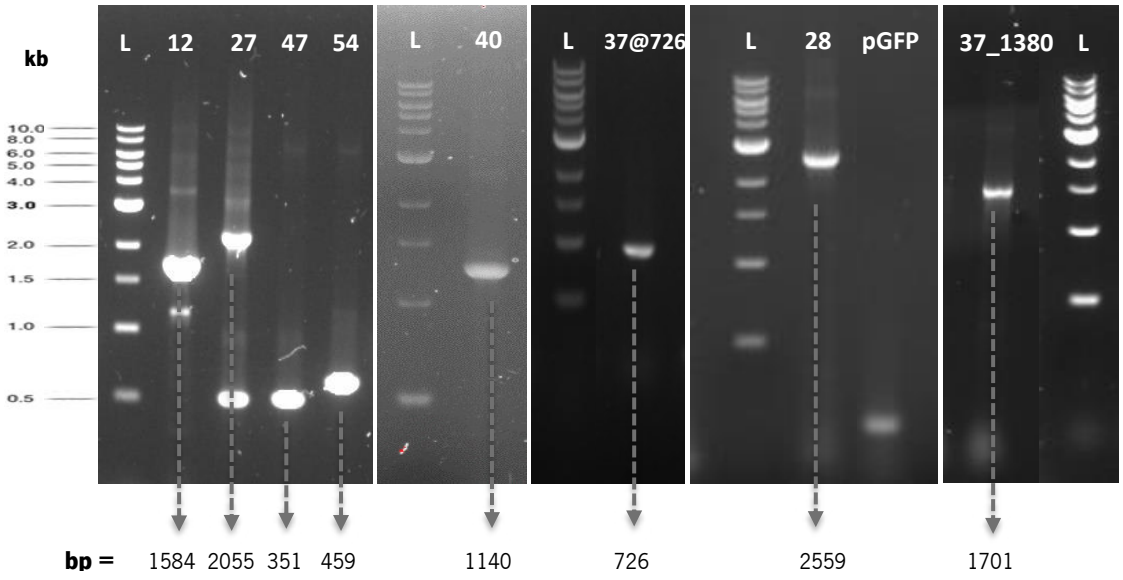

Figure 8- Colony PCR products in 1% agarose gels from positive recombinant TFP plasmids containing-colonies and empty plasmid used for TFP cloning, the pGFP plasmid containing colony as negative control. L is the 1kbp DNA Ladder (Neb) and for each positive colony the correct bands as well as its correct size are identified.

From the selected colonies a high percentage was observed to present an empty plasmid which can be a consequence of inefficient dephosphorization, the presence of kinases (that re-phosphorylate the vector after the AP action), incomplete cleavage by the restriction enzymes, or the insert sequences are not tolerated by the used *E. coli* strain, or ligation failure [127]. Indeed, when running the plasmid digestion in a gel, sometimes two bands were observed showing that the restriction enzymes were not able to digest all the plasmid in the reaction.

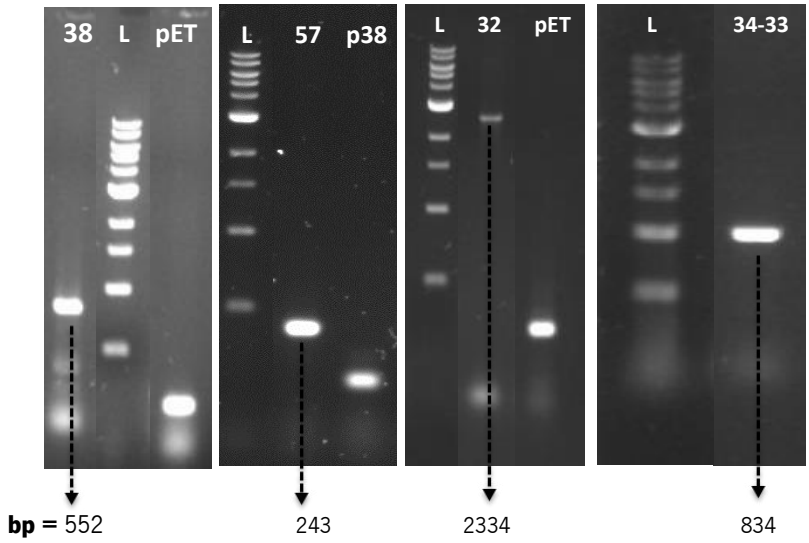

Figure 9- Colony PCR products in 1% agarose gels from positive recombinant chaperones plasmids containing-colonies and empty plasmids used for its cloning, the pCDFduet or pETduet plasmid containing colony as negative control. For g*57*, the constructed recombinant plasmid with g*38* chaperone was used and the primers in the colony PCR reaction were F90 and

R76. The DNA ladder was 1kbp DNA Ladder (Neb) and for each positive colony the correct bands as well as its correct size are identified.

Thus, to reduce the background colonies some optimizations were made, the incubation time of the digestion and ligation steps were increased above the time reported by the manufacturer's instructions. In the ligation step it was also increased the molar ration of the insert amount over the plasmid to increase the probability of target gene insertion into the plasmid, different T4 DNA ligases and a posterior digestion step with an enzyme that cuts only the empty plasmids were used (*NotI* for TFP constructions and *SacI* for chaperone constructions)

Besides the several attempts and optimizations to clone all the genes by traditional cloning, it was not possible to obtain any correct construction for the three biggest genes: g*28* with 2559 bp, g*37* with 3081 bp and g*34-32* with 3169 bp. This might be related to the inserts size that can cause plasmid instability placing constraints on its replication [128]. Consequently, the high-throughput cloning strategies CPEC and Gibson Assembly described in sections 2.3.2. and 2.3.3. were used to clone the PVP-SE2 chaperones (g*34-32*) and PVP-SE2 TFP (g*28*) genes. In line with *Quan and Tian* (2011), instead of plasmid linearization by PCR using primers to generate products with long overlapping sequences, the vector was linearized using *SacI* for pGFP and *NcoI* for pCDFduet. Even so, giving the difficulties in obtaining the correct construction, some optimizations were made: increase of the number of cycles recommended for complex constructs (for 35 instead of 20); dimethyl sulfoxide (DMSO) addition to reduce high GC%-rich primers effects; increase of the extension time (because of long length of genes); increase of insert molar ration over the vector; and the use of *DpnI* enzyme (that cuts metilated DNA template and thus reduces the background) [124,125]. These optimizations did not result in colonies with the correct construction and thus it was decided to test the use of a non-linearized vector instead of linearized plasmid, contrary to *Quan and Tian* (2011). With this approach a correct construction with g*28* was obtained as it can be visualized in Figure 9, but not for g*34-32* cloning. The Gibson Assembly technique did not result also and so, these chaperones were split into two sequences, g*34-33* and g*32*, and their cloning into the same (first g*34-33* and then g*32*) and separated vectors was attempted, with positive results only in the last approach. The recombinant correct constructions were used to transform *E. coli* BL21 (DE3) cells obtaining this way clones with TFP, and clones with chaperones. The *E. coli* BL21 cells with the chaperones were made competent to receive the plasmids constructed with the TFP obtaining the following combinations: TFP with g*57-38*, TFP with g*32*, TFP with g*34-33* and TFP with g*32* and g*34-33*.

In the case of cells simultaneously harboring the plasmids with TFP, g*32* and g*34-33* (confirmed by colony PCR), except for the TFP g*37@726*, they were not able to grow, even using less antibiotic concentrations than initially (25 µg/mL ampicillin, 30 µg/mL kanamycin, and 50 µg/mL spectinomycin). This behavior might be explained by the loss of at least one plasmid during cell replication because of plasmid instability or plasmids incompatibility (pGFP that is used for TFP and pETduet for g32, both with the same *ori)*. However, protein co-expression using plasmids with the same *ori* was already demonstrated with plasmid loss only after many generations [128–130]. Another explanation is the high pressure exercised by three different antibiotics, which according to *Busso et al* (2011), the co-expression of three individual constructions harboring each a different antibiotic into the same cells can lead to an prejudicial increase of burden on the cell [131].

3.2. TFP expression

The production of tail fiber proteins by recombinant methods is a process that can be associated with some difficulties, since these proteins usually have trimeric structures [121], and the problems related with their structure acquisition might be contoured by the presence of specific chaperones that help in the tail fibers proteins assembly and folding [132], allowing its soluble expression and binding ability to multiple receptors molecules [121]. Indeed, various studies report the need of chaperones for the correct expression of functional TFP [111,113,116,122,133]. The expression of the TFPs was thus performed with and without the co-expression of the three chaperones combination: chaperones of the *Myoviridae* T4 phage (gp57 plus gp38) and chaperones of the *Siphoviridae* PVP-SE2 phage (gp34-33 and gp32).

E. coli BL21 (DE3) cells harboring only the recombinant chaperones plasmids were grown and induced for protein expression to assess chaperones production. The expression was evaluated by SDS-PAGE and compared with *E. coli* BL21 (DE3) cells without the recombinant plasmids in the same conditions. The Figure 10 show a SDS-PAGE of the protein content for *E. coli* BL21(DE3) cells with and without the recombinant plasmid p57-38, when submitted to the same expression conditions. Comparing the protein profiles, it is evident the overexpression of a protein with a molecular weight of ≈26 kDa size (identified with the arrow) after the IPTG adding, that is absent in the protein profile of the same cells but without recombinant plasmid. However, this band does not correspond to any of the expected bands of gp57 or gp38 expression that should have approximately the predicted sizes of 8.991 kDa and 20.424 kDa, respectively. The overexpressed protein can be a result of a fusion between the two proteins since

the molecular size is close to the sum of the two chaperones (29.3 kDa), but that is not likely since a stop codon exists after gp57, separating the two proteins. The process of protein translation termination does not always work at 100 %, being naturally suppressed by several complex mechanisms, including the ribosomal frameshifting, suppressor tRNAs (aminoacylated tRNAs with anticodons complementary to STOP codons in mRNA) and STOP codon readthrough (RT)) [134], which in this case is a possibility. The non-observation of bands corresponding to the overexpressed proteins with the predicted sizes may also be a result of a low expression impairing their visualization on the SDS-PAGE, or being masked by other natural *E. coli* BL21 (DE3) proteins.

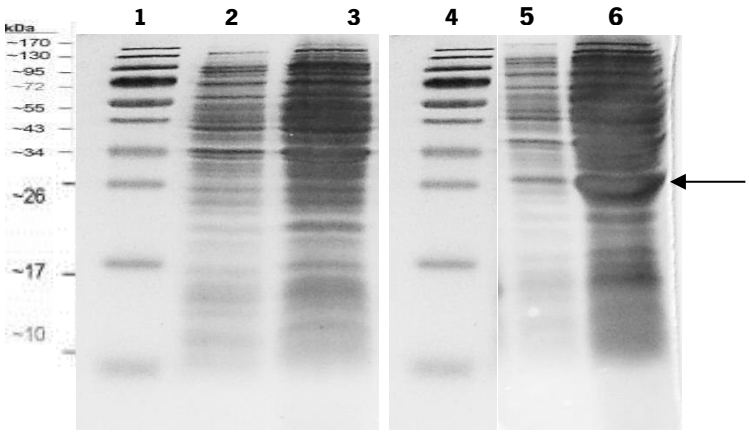

Figure 10- Protein expression profile for *E. coli* BL21 (DE3) cells without and with gp38+gp57 chaperones (in pETduet) after and before expression induction with IPTG. 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) cells without chaperones before IPTG adding, 3) cells without chaperones after IPTG adding, 4) cells with gp38+gp57 before IPTG adding, and 5) cells with p38+gp57 after IPTG adding. The arrow indicates the 26 kDa band resulted from recombinant chaperones expression induction.

Considering the existent research about the use of gp38 and 57 for TFP from T4 phage co-expression and that in any of them it was reported problems associated with the chaperones expression [111–113,116,122], in this study, it was attempt the expression temperature of 16 °C (according to the literature), but even in these conditions the same expression protein profile was obtained, with an overexpressed protein with 26 kDa.

The literature reports used different constructions approaches for chaperones and TFP cloning, the TFP (gp37 and gp12) were inserted in the same plasmid as gp38 and another plasmid was used for gp57 cloning [113]. Other authors used a cloning strategy similar to the one used here, cloning gp*37*, gp*38* and gp*57* in three different expression plasmids namely pET30a(+), pCDFduet, and pET57 [112]. Despite of the differences in the cloning strategy, the expression of the chaperones should be similar.

A problem that can be pointed in the strategy presented here is the use of plasmids with the same *ori*. To assure that this was not the problem the plasmid used for the chaperones cloning was changed from pETduet to pCDFduet. In the Figure 11 is represented the protein profile of p57-38 (in pCDFduet) expression by *E. coli* BL21 (DE3) showing the same protein profile with the same overexpressed protein with 26 kDa. This demonstrates that this protein is a product of the gp*57* and gp*38* genes expression.

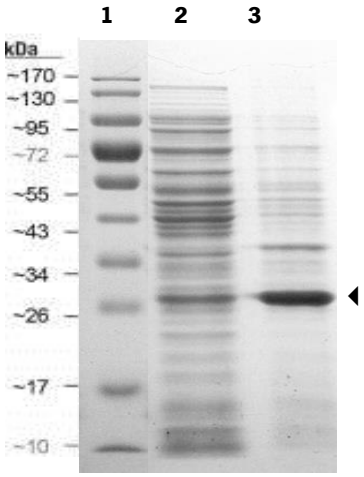

Figure 11- SDS-PAGE gel of *E. coli* BL21(DE3) cells harboring p57-38 (in pCDFduet plasmid) expressed at 30 °C, induced with 1mM IPTG. 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) gp57-38 pellet fraction, 3) gp57-38 filtrate fraction. The appearance of 26 kDa protein expression by these cells is marked by arrow.

The expression of the remaining chaperones by *E. coli* BL21 (DE3) cells where confirmed too, being expected an 18.8 kDa protein for gp34 and 14.2 kDa for gp33, for cells harboring p34-33 (in pCDFduet). As it can be visualized in Figure 12, two intense bands in both pellet and filtrate fractions exist that might correspond to gp33 and gp34, since it has one band lower than 17 kDa (the gp33) and another with a size close with 17 kDa that might be gp34. Also, it can be visualized than these two bands are stronger in the pellet than in the filtrate which shows that these putative chaperones are mostly expressed in insoluble form, demanding improvements in expression conditions to obtain more soluble protein. As it occurs with the gp57+gp38 chaperones fusion, the fusion of gp34-33 could have happened too, being produced a fused protein with a size that rounds the 33 kDa. In fact, it appeared a band with this size in the pellet and filtrate fraction of *E. coli* BL21 (DE3) harboring p34-33, yet this possibility was excluded since this 33 kDa band is present in all *E. coli* BL21(DE3) BL21 cells with and without recombinant product, meaning that this is not a product of gp34+gp33 expression.

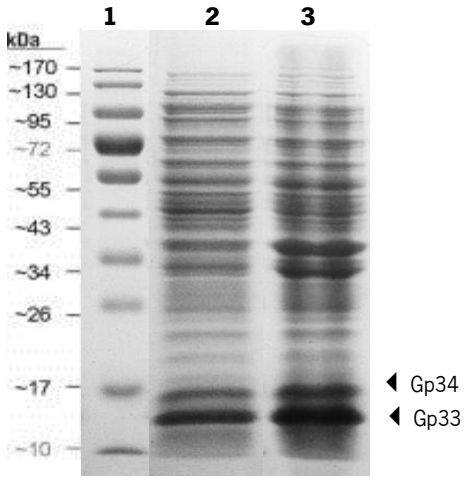

Figure 12- SDS-PAGE gel containing gp34-33 expression at 30 °C overnight with 1 mM of IPTG. 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) gp34-33 filtrate fraction, 3) gp34-33 pellet fraction.

For *E. coli* BL21 cells that hold the gp32 (in pETduet) chaperone it was also evaluated its expression at 30 °C overnight, induced with 1 mM of IPTG. Comparing the protein profiles from *E. coli* B21(DE3) cells with and without the recombinant plasmid before and after expression induction, a band with 86.36 kDa for gp32 was expected but an overexpression was observed only for a protein with a size between the 55-72 kDa (Figure 13). Although the size obtained is a little low than the expected it can still be a product of gp32 expression since this protein was not expressed by cells without the recombinant p32 plasmid when induced with IPTG. The reason for the smaller size could be a result from some protein processing/degradation by the *E. coli* cells.

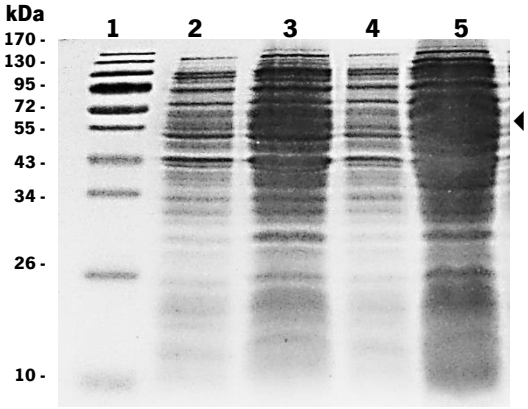

Figure 13- SDS-PAGE gel of proteins expression from *E. coli* BL21 cells without and with gp*32* containing plasmid. 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) cells without chaperone before IPTG adding, 3) cells without chaperones after cells disruption, 4) cells containing gp*32* before IPTG adding, 5) cells containing gp*32* after cells disruption.

Regardless of the chaperones expression difficulties, the TFPs were expressed with and without the chaperones. The PVP-SE2 and T4 TFP expressions performed by *E. coli* BL21 (DE3) cells without chaperones are represented in Figure 14, in which is visible for all filtrates a strong band corresponding to a soluble expression of the respective TFP fused with aceGFP, with the expected sizes (gp27- 102.63 kDa, gp37@726- 51.14 kDa, gp40- 68.78 kDa, gp47- 38.69 kDa, gp54- 43.18 kDa), except for gp12 that was not properly expressed in this attempt. However, the correct gp12 was obtained in other attempts. Besides the TFP expression by SDS-PAGE, the color of the cultures after expression was always an indicative of TFP expression, presenting an intense green coloration due to the presence of the aceGFP fusion protein.

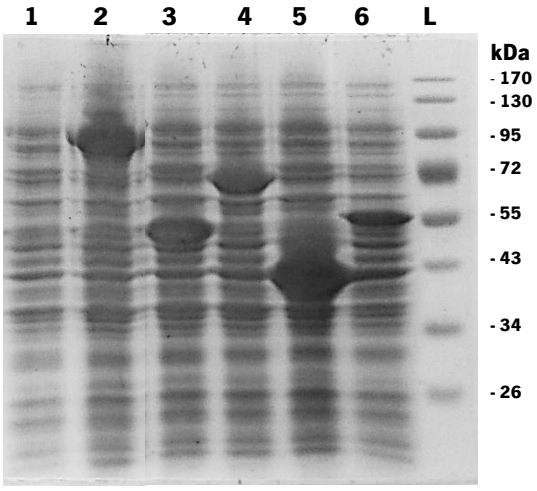

Figure 14- SDS-PAGE of filtrate fractions from PVP-SE2 and T4 TFP. 1) gp12, 2) gp27, 3) gp37@726, 4) gp40, 5) gp47, 6) gp54, L) EZ-Run™ Pre-Strained Rec Protein Ladder.

After the TFP expression alone, the co-expression with T4 chaperones (gp57-38) and with the PVP-SE2 chaperones (gp34-gp33) was accomplished.

The PVP-SE2 TFP co-expression with gp34-33 chaperones was successful, being obtained soluble TFP confirmed by the intense bands in SDS-PAGE gels and by the green filtrates color (data not shown), for each of the expressed TFP.

The expression of T4 TFP with the gp34-33 chaperones was not carried since these TFP (gp12 and gp37) were reported to need and express correctly with the gp57-38 T4 chaperones. The TFP from PVP-SE1 phage (gp40, gp41, and gp51) were co-expressed with gp34-33 chaperones, but because the culture after the expression did not present the green coloration typical from TFP expression fused with aceGFP protein, it was assumed that these proteins were not well expressed, which was confirmed by

SDS-Page gels that did not presented the bands correspondent to PVP-SE1 TFP expression (data not shown).

The absence of PVP-SE1 TFP expression was obtained too when co-expressed with gp57-38 T4 chaperones, as it can be visualized in Figure 15, except for gp51 TFP that presented a low intense band (marked with the arrow). For the others TFP, the expressions were not always consistent, since sometimes it expressed well (the filtrates presented green color) and other times failed. Even so, all the TFP were successfully expressed. The Figure 15 shows the co-expression of some TFP with the gp57-38 chaperones, being visible for some of them the correct TFP expression (marked with arrows).

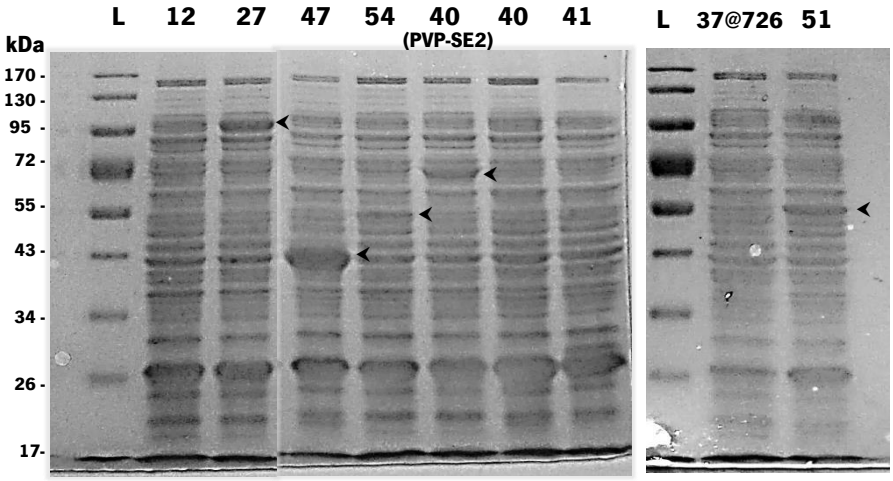

Figure 15- TFP co-expression with gp57-38 chaperones. L= EZ-Run™ Pre-Strained Rec Protein Ladder and arrows indicates the TFP soluble expression correspondent band.

The TFP were also expressed by *E. coli* BL21 (DE3) harboring the gp32 chaperone and its expression evaluated by SDS-PAGE (Figure 16). The figure shows the soluble protein content from TFP co-expressed with gp32, namely the gp51, gp54, gp40 (from PVP-SE2) and gp47 that clearly presented a strong expression proved by the large band presence with the correct corresponding sizes. The expression of gp41 from PVP-SE1 was present too, but in less quantity. The bands indicatives of TFP expression are marked by the arrows, not being marked any band in gp40 profile since it was the only one that was not expressed properly (the filtrate fraction was not green also).

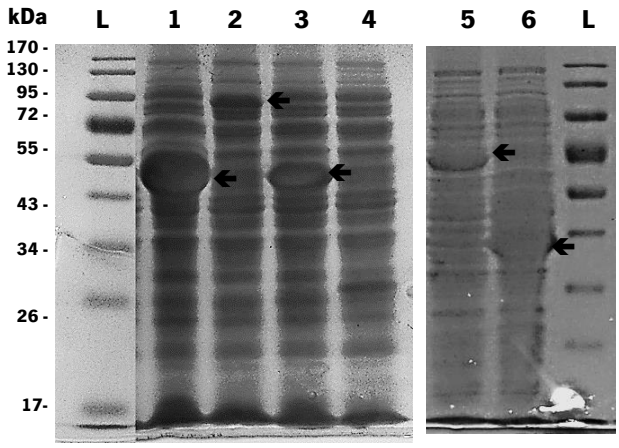

Figure 16- SDS-Page gel with the PVP-SE1 TFP (gp40, 41, 51) and PVP-SE2 (gp47, gp54, and gp40) filtrates from its co-expression with gp32 chaperone. L= EZ-Run™ Pre-Strained Rec Protein Ladder, 1) gp51, 2) gp41, 3) gp54, 4) gp40 (PVP-SE1), 5) gp40 (PVP-SE2), 6) gp47. The bands correspondent to the respective TFP are indicated with the arrows.

Since TFP gp28 was cloned lately, it expression was made alone, with T4 chaperones, and with PVP-SE2 gp34-gp33 separately from the other TFP expressions. The insoluble and soluble fractions of each gp28 expression can be observed in Figure 17, being always present a strong band with the correct size (≈121.8 kDa) correspondent to gp28 fusion with aceGFP protein. However, this TFP was obtained mainly in an insoluble form (in the pellet). Also, it was noted that the gp28 expressed amount was lower when co-expressed with the T4 chaperones, comparing with the expression level obtained by cells without chaperones and cells with PVP-SE2 chaperones, which might be a consequence of simultaneously expression of three proteins that in the others cells (without chaperones and with p34-33 chaperones) is not verified. Also, the expression of gp28 in the gp32 presence was attempted too but no expression level was observed.

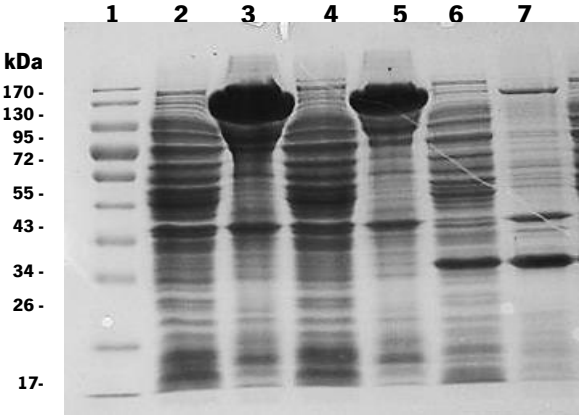

Figure 17- gp28 expression in *E. coli* BL21 (DE3) cells with and without chaperones: 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) filtrate from gp28 expression without chaperones, 3) pellet from gp28 expression without

chaperones, 4) filtrate from gp28 expression in *E. coli* BL21 (DE3) cells containing p34-33 chaperones, 5) pellet from gp28 co-expression with gp34-33 chaperones, 6) filtrate from gp28 co-expression with gp57-38 chaperones, 7) pellet from gp28 co-expression with gp57-38 chaperones.

The gp37_1380 was also expressed with and without T4 chaperones and a low intensity band was obtained at the predicted size (89.5 kDa) (Figure 18). Due to the low intensity, it is not possible to say that the protein was overexpressed.

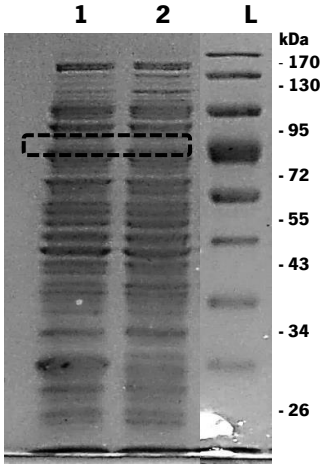

Figure 18- gp37_1380 expression in *E. coli* BL21 (DE3) cells with and without gp57-38 chaperones: 1) filtrate from gp37_1380 co-expression with gp57-38 chaperones, 2) filtrate from gp37_1380 expression without chaperones, L) EZ-Run™ Pre-Strained Rec Protein Ladder.

As referred previously, the only TFP able to co-express with the PVP-SE2 chaperones combination gp32 plus gp34-33 was the gp37@726, using the same expression conditions that were reported before. As it can be seen in Figure 19 most of the expressed TFP was retained in the insoluble fraction, with only a low amount present in the soluble fraction that was used for *E. coli* BL21 (DE3) posterior binding test.

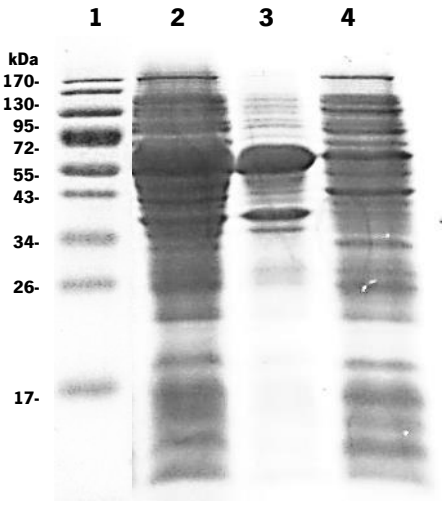

Figure 19- gp37@726 co-expression with gp34-33 plus gp32. 1) EZ-Run™ Pre-Strained Rec Protein Ladder, 2) gp37@726 co- expression with p34 -33 plus gp32 sample after lysis, 3) gp37@726 co-expression with p34 -33 plus gp32 pellet fraction, 4) gp37@726 co-expression with p34 -33 plus gp32 filtrate fraction.

The expression of recombinant proteins (chaperones, TFP, TFP co-expression with chaperones) obtained in this work is resumed in Table 15, which can be visualized the correct and incorrect expression for each one in terms of solubility and size.

Table 15- Resume of TFP expression results, in chaperones presence and absence, in terms of solubility and obtained size.

phages	Recombinant proteins	Expression		Co-expression							
		Soluble	size	gp57+38		gp34-33		gp32		gp34-33+gp32	
				Soluble	size	Soluble	size	Soluble	size	Soluble	size
T4	gp12	✔	✔	✔	✔	-	-	-	-	-	-
	gp37@726	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
	gp37_1380	No expression was verified									
PVP-SE1	gp40 gp41 gp51	Low expression level for all conditions									
PVP-SE2	gp27	✔	✔	✔	✔	✔	✔	-	-	-	-
	gp28	✘	✔	✘	✔	✘	✔	✘	✔	-	-
	gp40	✔	✔	✔	✔	✔	✔	✔	✔	-	-
	gp47	✔	✔	✔	✔	✔	✔	✔	✔	-	-
	gp54	✔	✔	✔	✔	✔	✔	✔	✔	-	-
T4	gp57+38	✔	✘	-							
	gp34-33	✘	✔								
PVP-SE2	gp32	✘	✘								
	gp34-33+gp32	-	-								

(✔): yes/ correct; (✘): no/incorrect; (-): not tested.

3.3. TFP binding ability

The filtrates from each TFP expression, with and without chaperones co-expression, were used to evaluate the attachment capability of the TFP to the bacterial host cells, proceeding as referred in section 2.8.

The tail fiber proteins from PVP-SE1 phage were tested both against *Salmonella* Enteritidis S1400 and *E. coli* (DE3) bacteria, since that this phage is able to infect both bacteria [106,108]. However, none

of the putative TFP could recognize the bacterial hosts, even when expressed in the *Myoviridae* (T4) chaperones and *Siphoviridae* (PVP-SE2) chaperones presence. The low/no expression level of these proteins or chaperones might be the reason, but it cannot be excluded the possibility of these not being the responsible for the specific recognition of the host, turning necessary the search and test of other putative TFP genes. Also, it is not certain if the tested proteins are or not involved in its host recognition, since it is possible that they might require the presence of other PVP-SE1 proteins that help in the TFP function or structure acquisition. Indeed, it is possible that the *Salmonella* recognition is made by more than one PVP-SE1 TFP that together form complex structures involved in the host recognition. Given the similarity to the T-even phages, the PVP-SE1 (a *Myoviridae* phage too) tail fibers might be composed by complex structures comprising several different proteins [135]. Also, it is not known if the non-binding ability of the tested PVP-SE1 TFP is related to the expression of them fused with aceGFP protein that might change the protein conformation leading to an inefficient exposure of the domain responsible for the *Salmonella* host recognition, since the fusion partners can potentially interfere in the correct structure and function of recombinant protein [136–138].

The TFP from T4, already described to be able to bind to *E. coli* BL21 (DE3) when co-expressed with gp57 and gp38, were also tested as a control of the approach used to assess the TFP attachment ability. Thus, the gp12 and gp37@726 were tested against *E. coli* BL21 (DE3). gp37@726 was not able to decorate/bind to the *E. coli* cells when observed under the fluorescent microscope, independent of the used chaperones for co-expression. As referred before, gp37 was already tested and reported to be able to bind to *E. coli* bacteria [112,113]. Although, the g37@726 corresponds to a gp37 truncated version (since it was not possible to clone the entire gene) and this can lack some important amino acids essential to recognize *E. coli* bacteria. Also, the inefficient gp37@726 attachment might be a result of a TFP poor expression or inadequate assembly by gp38 and gp57 chaperones that due to its improperly expression might not turn the protein functional. Moreover, since that the gp57 and gp38 might not have been expressed properly, it is unknown if the non-binding was a result of the TFP correct structure acquisition due to the chaperones absence or by the aceGFP, fusion partner that impaired binding.

In opposition to the gp37@726 results, the gp12 short TFP when tested against *E. coli* BL21 (DE3) could recognize the cells independently of being expressed with or without the gp57 and gp38 chaperones presence. Under the fluorescent microscope, it was possible to observe well individualized and contoured bacterial cells decorated with the fluorescent gp12 (using a short exposure time= 254 ms) that matched exactly with the bacterial cells visualized in bright field (Figure 20).

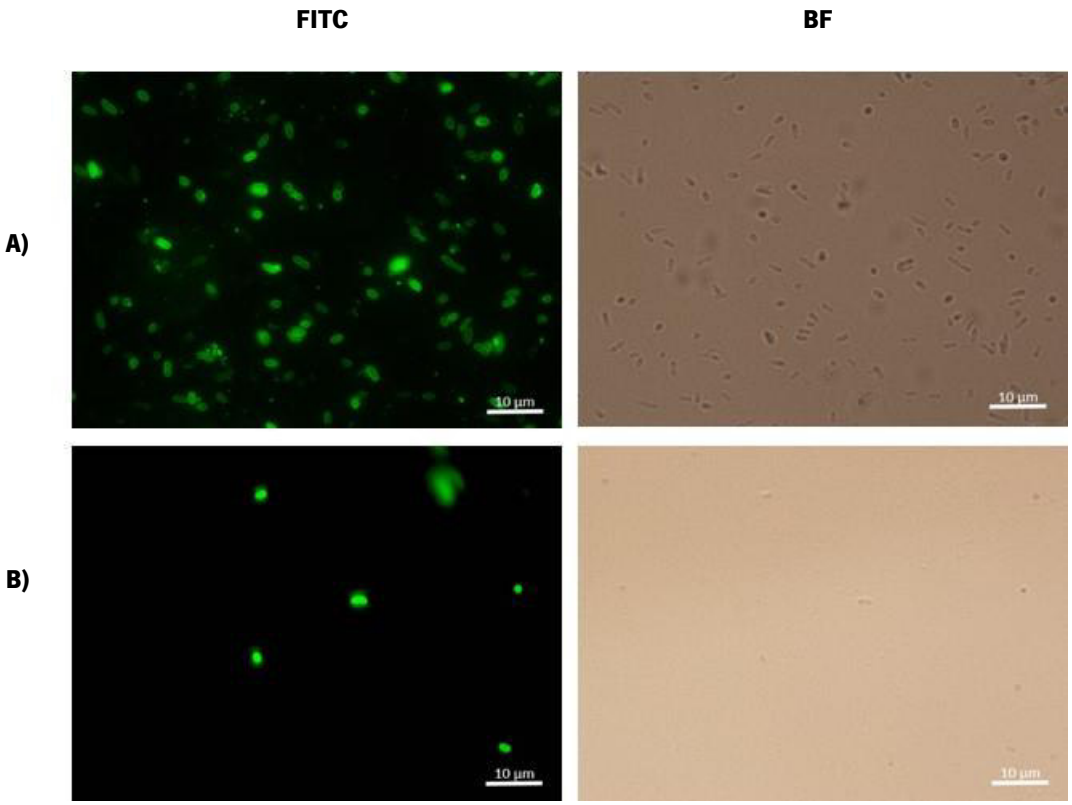

Figure 20- Fluorescent microscope images obtained for gp12 binding to *E. coli* BL21 (DE3). **(A)** Images for gp12 expressed in chaperones absence, **(B)** Image for gp12 co-expressed with T4 chaperones.

The binding of gp12 to the bacterial cells when expressed in the absence of T4 chaperones showed that, contrary to what has been reported [111,116,117,121,122], the binding of this short TFP is independent of the trimeric structure that is usually mediated by the gp57 chaperone (or buffers that lead to the monomers folding into homo-trimers). Although suggesting the need for the trimeric structure for the TFP to be functional (and they proved that gp57 was required to obtain the trimers), the previous studies did not test the binding ability of the soluble dissociated monomers of gp12 to *E. coli* cells.

The gp37_1380 was not tested against *E. coli* cells since it was the last one to be cloned and it was only possible to expressed it once, in its great majority as an insoluble protein.

Despite the negative results for the truncated gp37, the positive results obtained for the gp12 short T4 tail fiber protein showed that the used strategy (using the aceGFP, protein as reporter and visualization under the fluorescent microscope) is successful to functionally analyze binding proteins able to recognize bacterial cells.

The same strategy was used for the putative TFP of PVP-SE2 which enabled the identification of the binding ability of gp27. This protein, expressed alone or with the PVP-SE2 chaperones, could recognize the *Salmonella* Enteritidis 821 strain (the PVP-SE2 phage host) as can be seen in Figure 21.

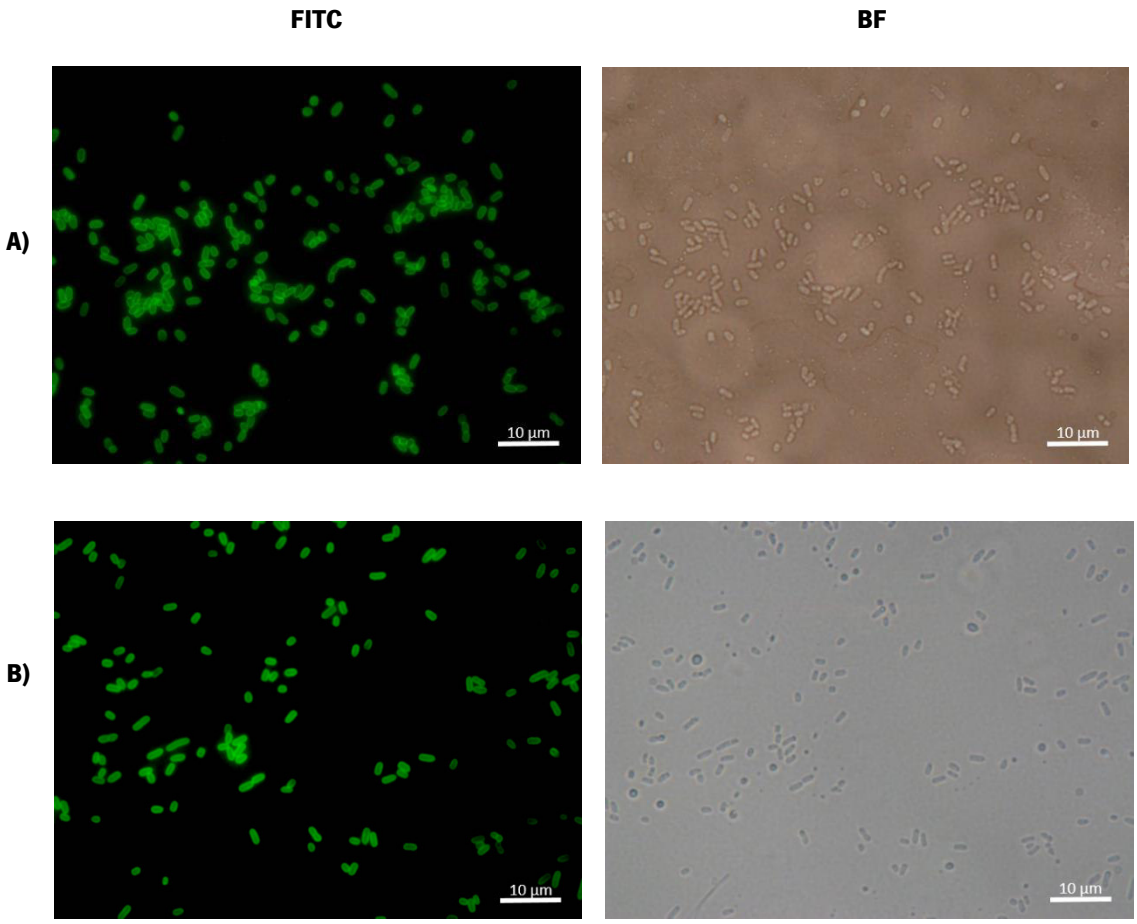

Figure 21- Fluorescent microscope images obtained for gp27 binding to *Salmonella* Enteritidis 821 when the protein was expressed in gp34-33 presence and absence. **(A)** Image for gp27 expressed in chaperones absence, **(B)** Image for gp27 co-expressed with gp34-33 chaperones.

Also in this case, the TFP did not required the co-expression of chaperones for the protein to present its binding activity. The remaining TFP proteins from PVP-SE2 were not able to bind and recognize the *Salmonella* phage host, even when expressed in the presence of chaperones. Unlike the T4 phage (*Myoviridae*) which presents two proteins able to bind to the host (short and long tail fibers) [109,110,112,113,122] phage PVP-SE2 (*Siphoviridae*) seems to present only one protein responsible for the phage recognition of its host.

As a negative control, gp27 binding ability was tested against *E. coli* BL21 (DE3) cells. Knowing that the PVP-SE2 phage does not infect *E. coli* cells [106] and that the TFP are the responsible for the phage-host recognition, it was expected that the gp27 should have the same behavior as the whole phage. In fact, gp27 was not able to bind to *E. coli* confirming its main role on phage adsorption.

Consequently, to be use in further studies, gp27 was purified by a HisPur™ Ni-NTA Resin with affinity to histidine tag as described in section 2.6, and the wash, flow through and elution's fractions

were loaded in a SDS-PAGE to assess the purification step. The Figure 22 shows the SDS-PAGE gel with gp27 purification fractions, being visible that a high quantity of recombinant protein remains in the flow through and that minimal contaminants are still present in the elution (300 mM). The purified protein was posteriorly concentrated and quantified obtaining a concentration of 0.766 g/L, i.e., 63 μM.

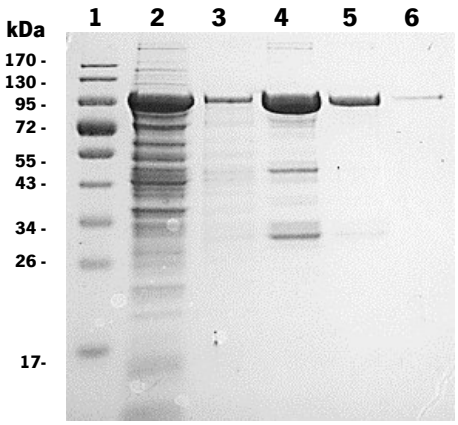

Figure 22- SDS-PAGE of gp27 fractions during purification. 1) EZ-Run™ Pre-Stained Rec Protein Ladder, 2) Flow through, 3) Wash, 4) elution with 100 mM imidazole, 5) first elution with 300 mM imidazole, 6) second elution with 300 mM imidazole.

To understand the level of oligomerization of the TFP, gp27 expressed without chaperones and functionally active, were analyzed by polyacrylamide gel electrophoresis in non-denaturant conditions. The Figure 23 shows the differences between the native and denatured gp27 expressed without chaperones, when loaded in an 8% polyacrylamide gel without SDS and at cold temperatures to avoid the native form denaturation. It is visible that the gp27 TFP in its native form has a conformation that gives it ability to bind to *Salmonella* host, and that consist in higher molecular weight, above the 170 KDa. This size is consistent with the existence of gp27 dimers. Although, the suggested conformation of gp27 TFP in dimers cannot be concluded just by the size obtained in the native polyacrylamide gel, since that others parameters like weight, charge and shape influence the protein mobility [139].

The same was done with the gp12 from T4 phage in order to understand if the protein expressed without the T4 chaperones has the same structure as when co-expressed with the gp57 chaperone, being the reported in this case to be a parallel homotrimer that in its native form is resistant to dissociation with SDS at ambient temperatures [116,117,122]. As it can be seen in Figure 23, there is no difference between the native and denatured gp12, existing in both a low intense band that might correspond to gp12 monomers with a size that rounds the 83,6 kDa when fused with aceGFP protein. This demonstrates

that gp12 expressed in this work without the gp57 and gp38 chaperones presence, is in the form of monomers.

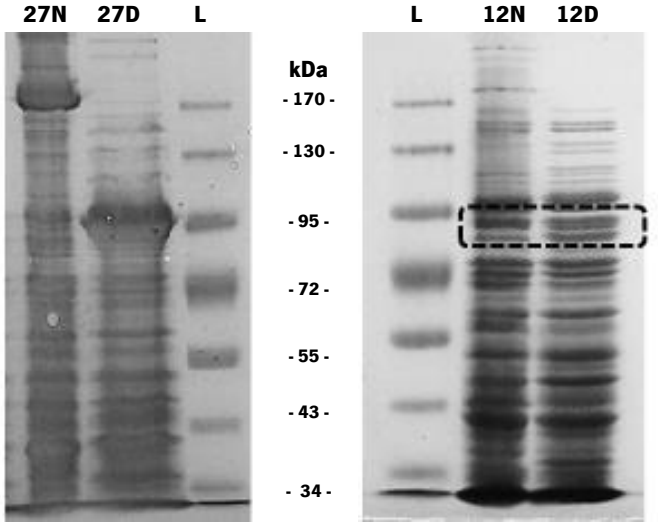

Figure 23- 8% polyacrylamide gel in non-denaturant conditions of both native (N) and denatured (D) protein content from filtrate fraction of gp27 and gp12 expressed without chaperones. L= EZ-Run™ Pre-Stained Rec Protein Ladder.

These results are contrary to the literature, which describes that this TFP when expressed with chaperones acquire a trimeric structure that is mainly present in insoluble fraction [111,113,116,121,122]. Also, the dissociated monomers spontaneously refold into the native trimers when exposed to specific buffers, as well as it renatures after the gp57 purified adding. Thus, the trimeric structure of gp12 can be achieved in the presence or absence of gp57 by applying specific buffers that renatures the aggregated gp12 monomers. However, the co-expression of gp12 in the presence of gp57 is reported to yield in high soluble trimers compared with the quantity obtained in the absence of this T4 assembly chaperone [121,122]. This chaperone is thought to suppress the short T4 TFP monomers aggregation before its trimerization, which is important when the TFP is overexpressed since that in high concentrations the monomers have high tendency to aggregate, limiting the protein refolding. This kinetic is altered by the gp57 chaperone that is not necessary with low gp12 expressions, since that in low concentrations, the gp12 has ability to refold itself [110,116,121,122].

The literature reports the application of gp12 for *E. coli* detection only when in trimeric structures presupposing that the dissociated monomers were not able to bind and recognize bacterial cells. Nevertheless, in this study it was proved that the dissociated soluble gp12 monomers are enough to

recognize *E. coli* cells, without the need of the trimer structure acquisition that requires the presence of gp57 chaperone or others refolding strategies.

Considering the ability of gp27 to bind to the *Salmonella* phage host and the phage lytic spectrum, the TFP specificity was tested using different *Salmonella* strains, including different serotypes and subspecies (mostly *Salmonella* enterica subsp I which are the most common) and the results are represented in Table 16.

The recombinant gp27 was able to recognize the same *Salmonella* bacteria that the phage infects and the ones that are lysed from without [106]. The strains that the TFP does not bind are the ones that the phage does not infect (or lyse from without). These results corroborate that the gp27 is the receptor binding protein of phage PVP-SE2, the responsible for the specific host bacterial recognition, apart from *Salmonella* Typhimurium NCTC 12416 subsp. I, which was recognized by the recombinant gp27 but is not included in the PVP-SE2 phage host range. From all the *Salmonella* Enteritidis tested, only for *Salmonella* Typhimurium NCTC 12416 subsp. I the gp27 binding was not coherent with phage infection, and *Salmonella* Enteritidis SGSC 2474 was the only which the gp27 did not bind. The gp27 could identify ≈ 68 % of the tested Salmonella subsp. I, not being able to recognize the remaining *Salmonella* subspecies.

The *Salmonella enterica* subsp. I is a vast group that includes almost all the *Salmonella* responsible for warm-blooded animals infections [140] and thus an important target group for efficient detection. The diversification among the group makes difficult to target them with only one recognition element however, with the combination of more recognition elements it might solve the *Salmonella* detection problem. Indeed, the use of a single tail fiber protein (or other recognition element) able to specifically recognize the entire *Salmonella* genus would be remarkable (and very unlikely).

Table 16- gp27 protein from PVP-SE2 phage ability to bind to several *Salmonella* strains and PVP-SE2 infection ability for the same strains.

Enteritidis Strains	**Phage**	**gp27**	***Salmonella* subsp. I strains**	**Phage**	**gp27**	***Salmonella* subspecies**	**Phage**	**gp27**
AL55	✔	✔	Brandenburg SGSC 2460	✔	✔	SGSC 2425 subsp. II*	-	✔
EX2	✔	✔	Cholerasius SGSC 2461	✔	✔	Setubal SGSC 2567 subsp. II*	-	✘
S1400/94 (PVP-SE1 host)	✔	✔	Decatur SGSC 2465	✘	✘	Salamae SGSC 3039 subsp. II*	-	✘
269	✔	✔	Derby SGSC 2467	✔	✔	SGSC 3068 subsp. IIIb	✘	✘
546	✔	✔	Dublin SGSC 2470	✔	✔	SGSC 3069 subsp. IIIb	✘	✘
629B	✔	✔	Emerk SGSC 2477*	-	✔	Houtenae SGSC 2428 subsp. IV*	-	✘
657	✔	✔	Gallinarum SGSC 2423*	-	✔	Flint SGSC 2554 subsp. IV*	-	✘
821 (PVP-SE2 host)	✔	✔	Infantis SGSC 2483*	-	✘	Argentina SGSC 2555 subsp. IV*	-	✘
855	✔	✔	Montevideo SGSC 2488*	-	✘	Brookfield SGSC 2557 subsp. V*	-	✘
869	✔	✔	Muenchen SGSC 2490*	-	✘	SGSC 3100 subsp. V	✘	✘
905	✔	✔	Naestved SGSC 3612*	-	✔	Indica SGSC 2430 subsp. VI*	-	✘
932	✔	✔	Panama SGSC 2497*	-	✔	Ferlac SGSC 2581 subsp. VI*	-	✘
9510.85	✔	✔	Paratyphi B SGSC 2504*	-	✔	Vrindaban SGSC 2582 subsp. VI*	-	✘
NCTC 13349	✔	✔	Pullorum SGSC 2508*	-	✔	SGSC 3116 subsp. VI	✘	✘
ATCC 13076	-	✔	Reading SGSC 2510*	-	✔	SGSC 3118 subsp. VI	✘	✘
SGSC 2476	-	✘	Saint Paul SGSC 2513*	-	✔			
SGSC 2474	-	✔	Senftenberg SGSC 2516*	-	✘			
			Thompson SGSC 2519*	-	✘			
			Typhimurium NCTC 12416	✘	✔			

(-) strains that were not tested for phage infection ability.

Considering that these results are very preliminary and that more strains should be test, gp27 presents high potential to identify a high number of *Salmonella* serotypes belonging to the subspecies I.

The most prevalent serotypes responsible for illness are the Enteritidis, Typhimurium, Javiana and Newport according to the CDC 2014 annual report [141]. The present study showed that gp27 could recognize the two first serotypes. Thus, the gp27 TFP from PVP-SE2 phage is a promisor recognition element for future *Salmonella* detection methods.

Considering the potential gp27 application, it was intended in this work determine the minimal gp27 concentration necessary to decorate and visualize the bounded bacteria under the fluorescent microscope, using the *Salmonella* Enteritidis 821. A minimal concentration of 5 μM was found to be necessary for a properly tail fiber protein-host visualization under the fluorescent microscope with a low exposure time.

Considering that the bacterial receptor that is recognized by *Salmonella* phages is usually the LPS molecule, it was intended to determine if the *Salmonella* receptor that is recognized by PVP-SE2 phage is also the LPS and which part of the LPS. Thus, the PVP-SE2 phage ability to infect several *Salmonella* Typhimurium LT2 LPS mutants (proceeding as described in 2.8) was determined. Like PVP-SE1 phage [108], the ability of PVP-SE2 phage to lyse the LPS mutants was negative for the Rd1 and Rd2 mutants, being able to infect all the remaining mutants except for the wild type *Salmonella* LT2 for which lysis from without was observed, i.e., the phage could adsorb to cells but do not infect the bacteria (Table 17). Considering the size, limpidity and number of PFU/mL results, it can be seen that the infection efficiency of Re mutant by the PVP-SE2 phage is higher compared to the obtained for the remaining mutants. This suggests that, similarly to the PVP-SE1, the PVP-SE2 true receptor is the LPS inner core region [108].

Although, the ability of gp27 TFP to bind to the mutants was not very consistent with the phage infection ability, since the TFP attached well to the LT2 (wild type), then for the two next mutants (Ra and Rb1) the ligation was very weak, and for Rd1 and Rd2 mutants there was no binding. For the remaining mutants Rb2, Rb3, Rc and Re the ligation was strong, especially for the last two. This indicates that similarly to the phage, the gp27 has high attachment affinity for the mutants that lack the LPS outer core region, contrary to the Felix 01 phage [106,142]. Similarly to T7 phage, PVP-SE2 seems to infect better the rough *Salmonella* LT2 compared with smooth *Salmonella* LT2*,* in which the last ones the phage access to inner LPS core region is prejudiced by the outer LPS core [143]. Thus, besides the little divergences between the phage and gp27 results, it can be hypothesized that the PVP-SE2 host recognition is mediated by the tail fiber protein gp27.

Table 17- PVP-SE2 lytic infection of *Salmonella* Typhimurium LT2 LPS mutants and TFP binding ability.

Mutants	Phage plaques		PFU/mL	gp27 Binding
	Size	Limpidity		
LT2 (wild type)	-	-	Lysis from without	✔ ✔
Ra	+	+	7×10^6	✔
Rb1	++	+	12×10^6	✔
Rb2	++	+	7×10^6	✔ ✔
Rb3	+++	+++	8×10^6	✔ ✔
Rc	+++	++	8×10^6	✔ ✔
Rd1	-	-	-	✘
Rd2	-	-	-	✘
Re	+	++++	98×10^9	✔ ✔ ✔

Chapter 4

Conclusions

Salmonella is a foodborne pathogen that is still a serious worldwide concern for the public health, food industry, agriculture and veterinary, that demands for new approaches for it detection, control and therapy. This pathogen detection in food and water must be improved, to assure the consumers safety, by the advent of new pathogen diagnosis techniques that can overcome the traditional methods limitations.

The cornerstone in the development of new detection methods is the recognition element used that must be specific, stable, safe, accurate, have a low cost and easier production, able to distinguish viable from nonviable cells, recognition of small pathogen quantities, among others. The TFP from *Caudovirales* phages are crucial elements in the phage adsorption to bacterial host, being involved in the specific recognition of receptors exposed in the bacterial surface. Consequently, TFP are very promising as tools for pathogen diagnosis, with added advantages relative to the whole phage application, since the specific recognition capability is maintained, but allows the higher and low cost production, are stable, less susceptible to mutations, its properties can be easily improved by genetic engineering, there is no safety concerns on virulence genes and on the release of intracellular toxic components related to the bacterial lysis, among others.

Considering the pathogenicity of *Salmonella* and its constraints in foodstuff, the recognition element must detect the great majority strains of this genus. This demands for TFP from a broad host range phage origin, or multivalent, which is rare in nature.

The multivalent *Salmonella* lytic phage PVP-SE1 previously characterized by our research group is a promising tool for *Salmonella* control, detection and therapy. The putative TFP of this phage were thus used in this project to study their ability to recognize *Salmonella* after heterologous recombinant expression when fused to a green fluorescent protein, with and without the aid of chaperones. Unfortunately, any of the chosen putative TFP could recognize the *Salmonella* host, which might be related to several causes, including the lack of a correct structure/folding that hindered the binding domain interaction with the bacterial receptor or even the complete protein functionality. If this is the case the problem may rely on the chaperones: chaperones were not correctly expressed, did not performed their function, or other chaperones may be needed (specifically the ones from PVP-SE1 which have not been identified yet). Another possibility for the failure on TFP binding is the need of other proteins, even structural, to assembly a functional TFP. Moreover, considering the non-conserved nature of the TFP, the functional TFP may not be any of the selected proteins and not have been identified yet.

The successful expression of the short T4 tail fiber protein (gp12) fused with the aceGFP protein and its efficient binding to *E. coli* cells proved that this is a valid strategy. Moreover, the binding ability of

these expressed soluble monomers proved that, contrary to previous studies about this adhesin, it is not required the formation of trimers of gp12 for host recognition and consequently it is not required the use of universal gp57 assembly chaperones.

The validated approach enabled the identification of gp27 as a TFP with the ability to bind *Salmonella* cells, probably the unique from PVP-SE2, and it could bind even when expressed without chaperones. The protein expression (without chaperones) resulted in a soluble fraction able to fold into a conformation that seems to be into dimers. Comparing the binding spectrum of gp27 and of the phage adsorption it can be concluded that gp27 is the responsible element for PVP-SE2 host initial recognition (considering the lysis from without) as well as the responsible for PVP-SE2 host range determination.

The gp27 demonstrated to be a promising tool in *Salmonella* detection, especially for the *Salmonella* Enteritidis serotype which was always recognized by the protein, except for one strain (that still needs confirmation). Additionally, this recombinant TFP could identify 68 % of the different serotypes from *Salmonella* subsp. I, showing a powerful recognition ability for a high diverse and extensive bacterial group. It is worth to mention that *Salmonella* subsp. I, which includes the prevalent serotypes Enteritidis and Typhimurium, is the main responsible for the widespread and growing problem of foodborne diseases. However, more tests must be done for more accurate numbers on the protein specificity and the need for other(s) TFP with complementary spectrum is required to develop a diagnostic tool able to identify all the *Salmonella* subspecies.

This work also enabled the identification of PVP-SE2 and gp27 receptor on the *Salmonella* cell wall: the LPS inner core. This might explain the broad range among *Salmonella* (phage and LPS) since the inner core LPS is a much more conserved structure between strains. Considering the binding ability of gp27 and the agglutination common feature of TFPs it is possible to envisage an applicability beyond the detection tool, namely the *in vivo* use for pathogen clearance and inflammation reduction, something that was already demonstrated for gp12 from T4 phage and TFP from P22 phage.

As a general conclusion, this work showed that: the heterologous expression of putative TFP in fusion with a fluorescent protein is an efficient method for the functional analysis and specificity determination of the TFP; gp12 from phage T4 forms monomers during expression and does not need to be co-expressed with chaperones to be functionally active in its binding ability to *E. coli*; the identified TFP from PVP-SE1 are not able to bind *Salmonella* in the condition that were expressed (a correct expression of chaperones or other chaperones are needed); gp27 from PVP-SE2 is a functional active TFP responsible for the phage recognition and adsorption and presents high potential in the development of

a diagnostic tool for *Salmonella* and consequently can contribute to food safety in the combat of foodborne diseases.

Chapter 5

Future perspectives

At the end of this project several questions remain to answer, starting by the T4 gp37 TFP. This TFP needs to be cloned in its complete length and test its functionality when expressed alone to confirm the lack of its binding ability. The cloning of gp37 may be achieved by usual cloning or through other cloning techniques (using of commercial kits and plasmids that allow the easier insert cloning, like CPEC, Gibson assembly, Ligation independent cloning-LIC, In-Fusion technology, and others) [144]. If so, it must be co-expressed with chaperones gp57 and gp38 (using the already constructed plasmid). If functional it is confirmed the correct expression of the chaperones but if not, chaperone expression must be optimized to validate the TFP expression in presence of chaperones as requirement for host recognition. The chaperone expression optimization may require using plasmids successfully used in other studies and cloning the two chaperones in separated plasmids, to access the T4 chaperones soluble expression. Also, some expression parameters may be tested: higher or less O.D at the induction moment [145]; for proteins with complicated folding and with aggregation tendency it must be expressed slowly which mean that lower temperatures (15-25 °C) and lower IPTG concentration must be used [144–146]; the media and additives as cofactors that increase the protein solubilizing and protein degradation inhibitors [144–147]. With the chaperone expression confirmed, PVP-SE1 TFP can be co-expressed with the T4 chaperones and assess their functionality. In the case of not being functional, chaperones from PVP-SE1 must be identified and tested for their function with the TFP, and/or other potential TFP need to be identified. Also, if the non-binding of PVP-SE1 TFP persists, these putative genes can be expressed with other fusion partners or tags, to verify if aceGFP difficult the proteins-host interaction, for example the fluorescent partners mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry, among others [131,136–138,146–148].

Regarding the successful identification of the PVP-SE2 recognition element for *Salmonella* bacteria, further studies should be done for its characterization: *e.g.* the structure identification by X-rays crystallography, Circular Dichroism, NMR, SPR, among others; stability to pH, temperature, ionic strength, proteases, denaturants, and other parameters; binding domain characterization and the storage conditions.

The gp27 must also be tested against a higher pool of *Salmonella* bacteria to determine a more realistic binding spectrum of the protein. Other TFP from other *Salmonella* phages with complementary binding spectrum should also be investigated for the development of a diagnostic tool able to identify the great majority, if not all, *Salmonella*. Afterwards, the recombinant proteins will be applied as tool in different methods: biosensors, ELISA-based assays, flow cytometry or fluorescent microscopy to assess the improvement that these recognition elements may add to the methods. The chosen method will then

be assessed for its ability to detect *Salmonella* cells in food samples artificially contaminated and, lately, in naturally contaminated samples and compared with the golden standard (ISO 6579: 2003).

Chapter 6

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Annexes

Annex I: Competent cells preparation

a) Chemically competent cells by CaCl2 method

Using 1 mL from a pre-inoculum of 5 mL of LB (containing 1 bacterial desired colony from a fresh plate, grown overnight at 37 °C, 120 rpm of agitation), was performed an inoculum in 100 mL of LB media, and the culture was grown at 37 °C until reach an $O.D._{(600nm)}$= 0.3. After that, the cells were always manipulated on ice to not loose efficiency, being first pelleted (3300×g for 10 minutes at 4 °C). The pellet was carefully ressuspended in 50 mL of iced-cold 0.1 M $MgCl_2$, and then incubated on ice for 30 minutes.

Thereafter, the cells were centrifuged, and the pellet was ressuspended in 25 mL of iced-cold 0.1 M $CaCl_2$, and centrifuged again. The pellet was again ressuspended in 7 mL of 0.1 M $CaCl_2$ and the cells were pelleted again. Finally, the cells were ressuspended in 2 mL of 0.1 M $CaCl_2$ and it was added 150 μL of sterile 100% glycerol.

Aliquots of ≈ 80 μL were done and stored at -80 °C.

b) Electro-competent cells

Using 1 mL from a pre-inoculum of 5 mL of LB (containing 1 bacterial desired colony from a fresh plate, grown overnight at 37 °C and with 120 rpm of agitation), was performed an inoculum in 100 mL of LB media, and the culture was grown at 37 °C, 120 rpm until reach an $O.D_{(600nm)}$=0.3. The suspension was centrifuged at 3000×g at 4°c during 15 minutes and, keeping always on ice, the pellet was resssuspended in 40 mL of cold 10% glycerol, and the cells were pelleted again. Thereafter, the proceeding was repeated more three times ressuspending the pellets in a decreased volume of 10% cold glycerol, namely 20 mL, 10 mL and finally in 100-400 μL. The competent cells were aliquoted in volumes of 100 μL and stored at -80 °C.

Annex II: Restriction maps of the used plasmids

The plasmids used for TFP and chaperones constructions were all commercial expression vectors, with similar characteristics that can be visualized in Table 18 .

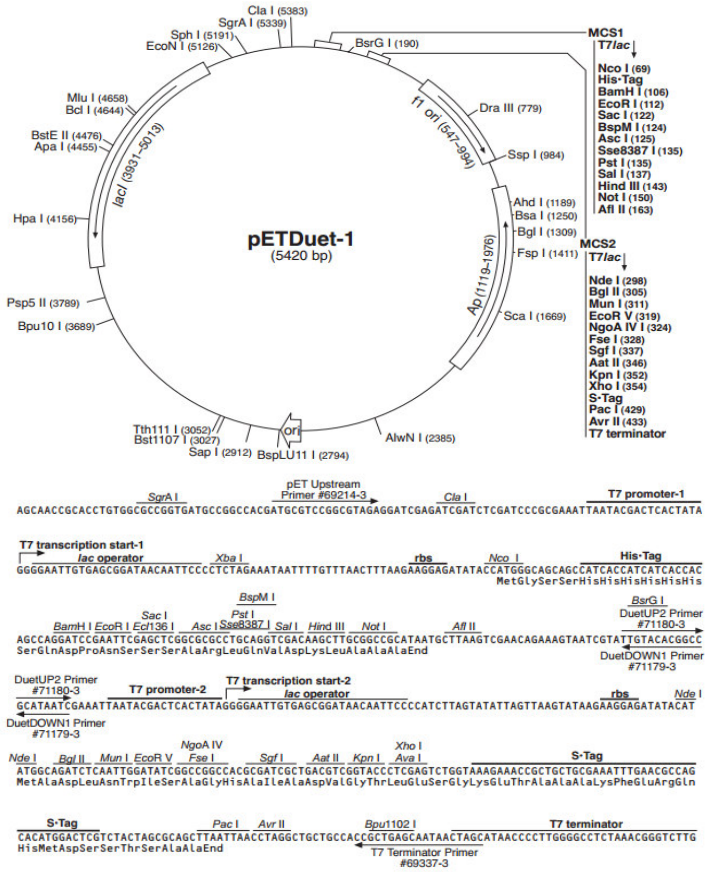
The restriction maps of used plasmids can be visualized in Figure 24 , Figure 25 , and Figure 26, and of which pETduet and pCDFduet are composed by two multiple cloning sites making possible to clone more than one gene in the same plasmid. On the other hand, pGFP and pET15b plasmids have only one multiple cloning site.

Table 18 -Main characteristics of used plasmids

plasmid	**Resistance marker**	**Promoter**	***ori***	**Purification**
pET15b	Ampicillin	T7*lac*	pBR322 origin	N' terminal His•Tag
pETduet	Ampicillin	T7*lac*	pBR322-derived ColE1 replicon	N' terminal His•Tag; C' terminal S• tag
pCDFduet	Spectinomycin	T7*lac*	CloDF13-derived CDF replicon	N' terminal His•Tag; C' terminal S• tag
pGFP (pET28a + *gfp*)	Kanamycin	T7*lac*	pBR322 origin	N and C' terminal His•Tag; N' terminal S•Tag;

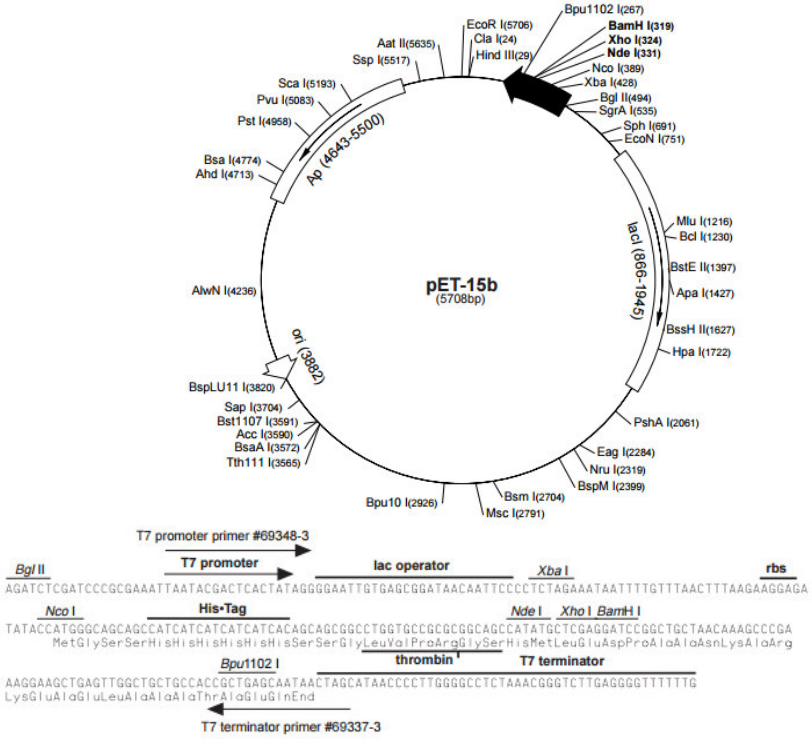
The Figure 24 and Figure 26 show also the sequence of multiple cloning sites for each plasmid, containing the enzymes that cuts in each local as well as primers that can be used for sequencing, promoters and terminators, and tags presents in C' and/or N' terminal. For pGFP plamid the *aceGFP* gene is present at the N' terminal of cloned genes in MCS that allows the posterior recombinant protein detection in fulorescent microscope. The sequence of each one of commercial used plasmids can be found online in Merck Millipore site as well as user protocols (available in http://www.merckmillipore.com/).

A)



pETDuet-1 cloning/expression regions

B)



pET-15b cloning/expression region

Figure 24- Restriction maps of chosen plasmids for chaperones cloning and expression as well as their multiple cloning site sequence. A- pETduet; B-pET15b.

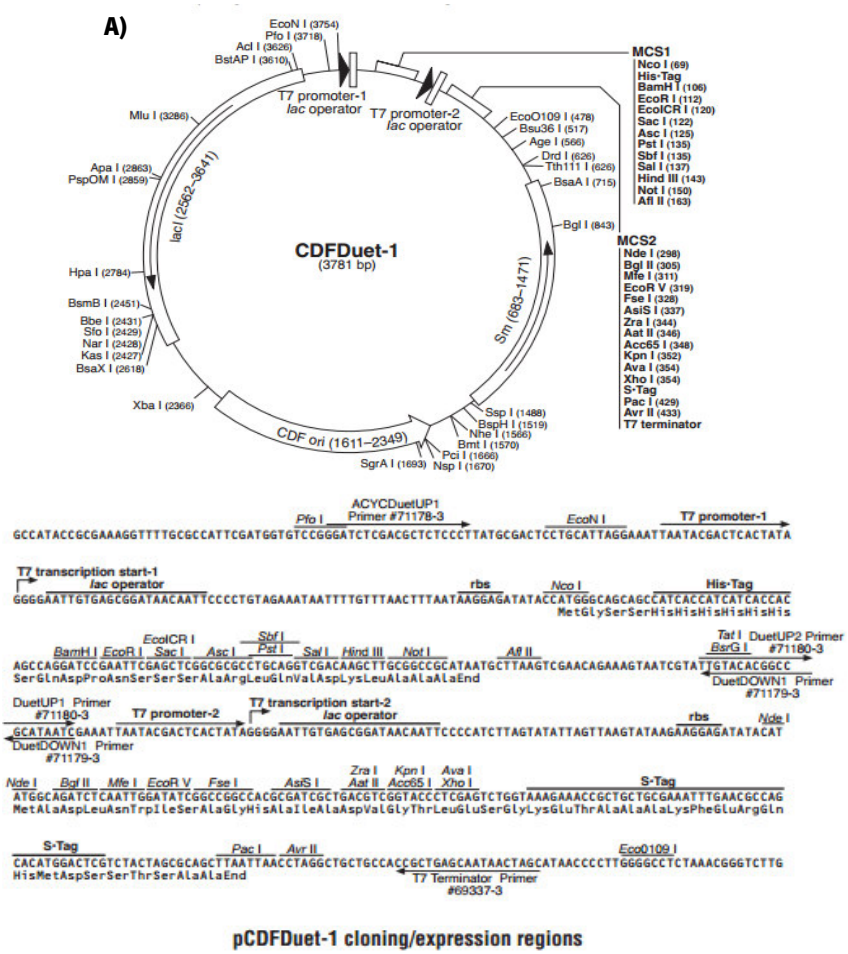


Figure 25- Restriction maps of chosen plasmids for chaperones cloning and expression as well as their multiple cloning site sequence. A- pCDFduet.

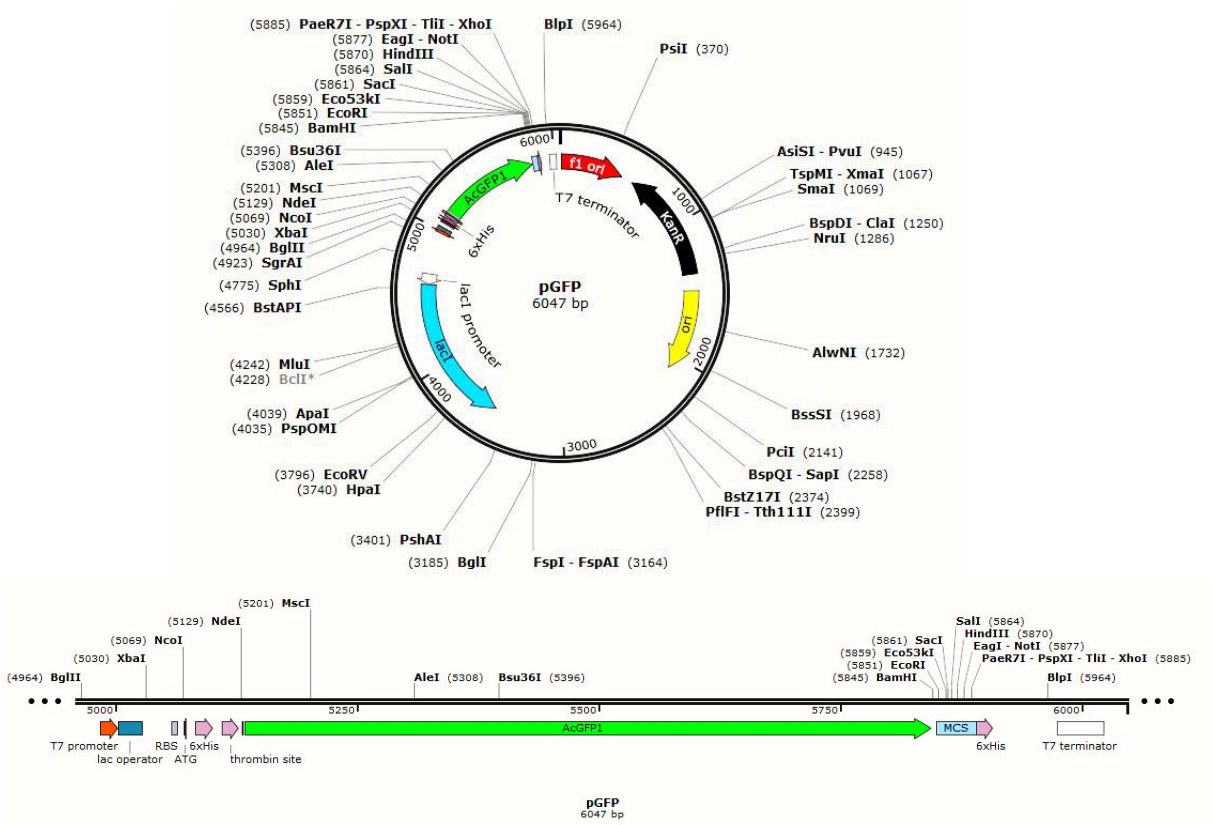


Figure 26- Restriction map of pGFP plasmid used for TFP cloning and expression, as well as the sequence of its MCS.

Annex III: T4 and PVP-SE2 nucleotide sequence

Table 19- Nucleotide sequence from PVP-SE2 genes

Genes	PVP-SE2
27	ATGTCCAGCGGTTGCGGTGATGTATTGTCACTTAATGATTTACAAATAGCTAAAAAACACCAGATTTTCGAAGCCGAGGTGATCACCGGTAA ACAAGGCGGTGTAGCTGGTGGTGCGGATATCGACTACGCCACTAACCAGGTAACCGGGCAGACGCAGAAGACGCTTCCCGCAGTCTTAC GTGATGCTGGTTTCTCCCCGGCATCTTTTAACTTTACGACCGGGGGTACTCTCGGCGTAAACGACGCGGATAAAGCGGTTCTTTGGCCGAA AGAAGATGGCGGGGACGGTAACTATTACGCATGGCGTGGCCCCCTGCCGAAAGTTATCCCTGCGGCGTCGACACCTCTTACGACGGGCG GCATTTCAGATTCCGCTTGGGTCGCGTTCGGGGATATCACCTTTCGCGCAGAAGCGGATAAGAAATTTAAATACTCCGTTAAGTTGTCGGA TTTTACCACTTTACAGCAATTAGCTGATGCTGCCGTCGACAGCGTTCTTATCGACCGTGACTACAATTTCAGCAATAACGAAACCGTTAACT TCGGCGGTAAGACCCTAACCATCGACTGTAAAGCGAAGTTTATTGGCGACGGTAACCTTGTTTTCACACAACTTGGTAGAGGGTCCGTTGT AGTTGGTGCTTACATGGAGAGTGTCACAACGCCGTGGGTGATTAAACCGTGGACTGACGATAATCAGTGGATAACCGACCCCGCAGCAAT CGTGGCCACACTTAAACAGTCTAAAACAGATGGATACCAGCCTACGGTAAACGATTACGCCAAGTTCCCGGGTATAGAATCCCTTCTCCCT CCGGAAGCTAAAGACCAGAACATTTCGTCTGTTCTTGAGATACGGGAATGTACAGGCGTCGAGGTTCACCGGGCGAGTGGTCTTATGGCG TGTTTCCTGTTCCGCGGATGCCATTTCTGTAAGATGGTAGACGCTGACAACCCGAGCGGCGGAAAAGACGGCGTAATCACCTTCGAAAACT TAAGCGGAGATTGGGGCAAAGGTAACTATGTTATCGGTGGGCGCACAAGTTACGGTTCGGTAAGTAGCGCGCAATTCTTACGCAACAATGG CGGTTTCGCGCGCGATGGCGGGGTCATCGGGTTTACCTCGTATCGTGCGGGGGAAAGTGGTGTTAAGACGTGGCAAGGTACGGTAGGTTC TACGACCTCTCGTAACTACAACCTGCAATTCCGGGATTCGGCGGTACTGTACCCTGTATGGGACGGCTTCGATTTAGGCGCAGATACTGAC ATGAACCCCGAAGATGACCGCCCAGGGGATTTCCCCATTTCTCAGTACCCGGTACATATGCTCCCATTAAACCATTTGATAGACAATCTAT TTGTTAGAGGTTCGCTGGGGGTAGGTTTCGGTATGGACGGGCAAGGTCTGTATGTCTCTAACATAACCGTCGAGGATTGCGCTGGTTCTGG GGCTTATATTCTTGCCCACGAAACAGTATTCACTAATATCGCAATAATCGACACCAATACTAAAAACTTCCCCGCGAACCAGATATATATCT CGGGGGCCTGTCGTGTAAACGGCCTTCGTTTGGTCGGAATACGGTCTACCAGCGAACAGGGCCTGACGATAGACGCACCTAACTCTACTG TAAGCGGAATAACGGGCTTCGTGGACCCATCAAGGATTAACGTAGCCAATTTGATGGAGGAAGGTCTTGGTAACTCGCGCATAAACAGTTT CAATAATGGTTCTGCGGCGCTTCGGTTTCGTATTCATAAACTGTCAAAGACCCTTGATAGTGGGTCCGTGTACTCCCACCTTAACGGAGGG CCGGGTTCTGGTTCAGCATGGACCGAAATTACCGCTATTGCGGGGTCCTTGCCTGATGCCGTGTCATTAAAAATAAACAGGGGCGATTATC GTGCTGTTGAGATACCGGTAGCGATGTCCGTCCTACCAGACAACGCTGTCAGGGATAACGGGTCTATATCACTGTATCTGGAGGGCGATA GCCTTAAAGCGTTAGTTAAGCGGGCCGACGGAAGCTATACAAGATTAACTTTGGCATAA
28	TTGGCGCTAGTAATCCACTATACCCGTAACGAAGACGGCACATTTGACGTTAAACGTTATCGCGATAATCCGATGAACTTCGTCGTGAACC ACGTTCCCGATGGGGTGCCGGTTCGCGTTTTCATCGACGAAATCGGGGAAGATAACGACGTAACAGAAGACTTCGAAGCACTGAAAGAAAA CGCGACTTTCCACATTGTGGAATCTGCCGGTGGTGGCGCTATTAAAGGCGTCATGAAGATTTTTAGCGTTATCCTTAAACCGCTAGCGAAA CTATTATCACCATCCGTGAAAGGGGCGTCATCTAACCTTGCGAACTCGCAGGCGGATTCCCCGAACAACAGTCTCACCGACCGTAACAAC AAGGCGCGCCCGTACGAACGCAGTTACGACATCTGTGGAACGGTGCAAACCATCCCCAATAACCTTATGTCTACTTACAAGGTGTTTAACG CCGCCGGTAAAATTGTAGAGTACGGCTATTACGACGCCGGGCGTGGATACCTCGACATACACCCTGAAGGTATAACTGACGGGGACACCC GCGTATCAGACATAACAGGCACGTCGGTTGCCGTGTACGCACCATATACATCGCCGAATAACACATCTACACCCCAGGTCATGGTCGGGG ACCCCATAGAGCAAGGCCTATACATTACCGTAGAATCTAACGAAGTTGACGGCGTGGTTCTTAAAGCACCGAACGGTCTGGGCATTTCTTT CTCTTACATGTCCGGGTATCCGTCTTTGTCCGGAAACATTGGCACGATATATGACCCAACAGGTGGTTCGGATTTTTCTGGGGTACTGGTG CCTAATGATACATTTTCGCTGGTGTCGGCGTGGACAAATACAGACGTTGACCTCTCCGGCGGCGGATATCAGGTAGTCAGCGTGTCCGAA GGGACCGTTACCTTTATCGTGCCTGGCGGCCTTATCGGGCGGTGGCAAGAAATAAGACCCGGTTCATTTTTCCGCGGTGATGGAGAGGCC TCGCTGCAACCAGACAACGCGTATGAGAAAACATTAACCGATTGGGTTTCAATAAACCGTACCGAGGTTGAGCGTATAGTCGCCAATATCG CCGCCGCGAACGGCATGTATAAAGACAACGGCAAATCGAAAACACTGGCGTCTGTAACCGCCGAAATACAGTATCAATTGCTCGATGAAAA CAGCACTCCTTACGGACCGATATATACTGCGCAAGGAACTGTGTCCGGGCGTACACCGGACTACAACGGCGTCACTATTTACGCCGACCT GCCGGTTGTGTCACGGGTGCGGGTGCGCGCCAGAAGGGTAACAGACCTGGACTTTAATTTCGAGGGGTCTGTAGTCGATGAGATAACGTA CGTTAACTTATACGGGCAAACACGTGACAACACCCCACACTACGGCAACAGAACAACAGTACACTCGATGCGCAAGCAGACCCCCCGTGC TGCGGAAGTAAAGCAACCGCAGTTGCGTATGATTGCTACTGAAATGGTGTACAAATACCTCGGTAATGGCGTTTTTGAAGACACGATGACC CCCAATACGCAAGCCGTGCAATCTCTTATCCGCCTGGCGCGTGATCCGGATGTGGGGGGTTTAAACCTGACGGTACGCAACATGGATAAG TTACTTGCTGTGCAGAACGAGGTCGAAGCGTATTTTGGCGACAAACAGGCTGGAGAATTTTGTTACACGTTTGATGACTATAAAACCACCAT GCAGGACATAGTTAGTACTATAGCAGACGCCATATTCTGCACCCCATATCGGCGTGGGGCGGATATCCTTCTCGATTTTGAGCGCCCTCG CATGGGCCCCGAGATGGTGTTCACCCACCGAAGCAAGGCCGGTACTTCTGAAAAGTGGACCAGAACATTTAACGATTCTCAGGTTTTTGAC AGCCTTAAATTCTCGTACATAGACCCTAAGACGAACGTCAAAGAGACTATAACCATACCCGAAACCGGGGGCCTTAAAACGGAGACTTACG ACTCAAAAGGAATCCGCAACTATAAGCAGGCTTTCTGGGCGGCAAACCGTCGCCACCAGAAGAACATTTTAAAGAAAATTTCGGTGTCGTT TACCGCCACTGAAGAGGGTATTTTTGCCCTTCCGAATCGTGCCGTTAGTGTGGTTAAGGGTTCGCGTATGTCTACTTACGACGGCTACGTA ACCGCGGTTAACGGTCTTACCGTAGAGCTATCCCAGCCGGTTAAGTTCACATCCGGAGATGACCATTATTTGGTTCTGAAGTTACGTGATG GCGGAGTCCAAAGTGTTCGTGTTGTCCCTGGCGCACATGACCGACAAGTAATTATGACGTCTGTGCCGCAAGAAGCCATTTACACTGGTAA TAGCGCTTTGAAAACTGAATTTTCATTCGGCAACGAAGCAAGGCATAATGCTCAGATGATTCTTGTTTCTACGGTAGACCCTGGCGATGACA GAACAGTCAAAATAACCGGGTTTAACTATGACAAGGATTTCTATAAGTTTGACAACGTGCCTCCTTTCGGTCGTGCGTTCTCCAGCGGATTC GATAACGGTTTTAACTAA
34-32	GTGGTTCCCGCCTGGCGCAACTACCAACCAGTAACCGACCAACCAAAGGGCATTACTATGAAACTTAGCGATTTTTACTACGAAGCCGAAG CCGAGAAAGGCGCGCGCATGCCGATCCCTTTAAAAGATGGTACAGATTCAGGAGAATGGTTGAACGTTGTCTCCCCGGAGGCCGATGTCG CAGTTAAAGCTATGCGTGCCTTCACCCTGGCGTACCGAGCGGCGGTAGGTAAATTAAAACCGCTTCGAGATAAATGCGAAGAGCAAAAAGA

	CTTCTCAGAATACAATTTAAAAATGGAAGACGCGGCAGGAGACCTAAACCGACAATTGGCTCTCGAATTGGTGAATGGCTGGAGTCTCGAT GATGAGTTCACTAAGGAAAATCTTAAGACTCTTCTCGCCCAATATAAGCGCCTGGCGGAACATGTAGTCGTATTCCACCACGAACAGTTGC GTCAATTGCAGGAAAAGTAGTTGTTTCAGTTTGCCCGTTGGAACTTCATAACCCGCCACGAAAGGCGCAAGTTTGACAGTATCGCCGATGG GCACAAAGCCGCGCTTATCGCTATGGGGGTAATAAAAGACGCGGGAGAAACAACGCAGGACGCCGGGCCTGAATGCCCCCCTGAACTAC TAACCACTTTTGAGAAGTATCGTGATGCTAAATTTACCCGCCGCGTCGATGACGACGGCGTAAAGCTATACCCAAGAGAGCAACTTAGTTG GTCAGATTTAGTGGCGTATAGCACTATTTCAGGTCAGAATATAGGGATGTTTGAATCTGAAATTATCATGGGCTTAGACGCCATTTTTGAGG GTAGAAACGATGGCTGAATGGCTGATGTAGCTAGCTTAGTAGTAAAGGTAACCGAACAAGGCGCGAAGGCCACATCAGACCGTCTTGATAA CCTCTCTAAATCTGCAAAAGTTGCGGGGGCCGCGGTCACCGGACTGGCTGCCGTTGTAGCCGCTACGGCCTATAAGGCGGCGCAGGAAC TAGTCGAGTCACAACGGCAACTGGATAAGATGTCGGCTAGCCTGAAAACACTCACAGGCAGTACTCAAGGCGCAAAACAGGCCCTGAGTA TTTTACAGGACTTCGCCCGTGACACACCTTACGGCCTTGAACAAGCAGTGGAAGGGTTCCGTAAACTGGTAGCTCTAGGCCTCACCCCAT CAGAAGAAGCGCTACGCTCTTACGGCAATACTGCATCAGCGATGGGTAAAGACCTTAACCAGATGATTGAGGCCGTCGCAGATGCGAGCA CCTTTGAATTCGAACGTCTGAAAGAATTCGGCATTAAAGCCAAGCAGAACCAAAGTGATATCGAATTTACCTTCCAGGGGACGACTACTGTA GTTAAGAAAAACGCTGCCGATATTGAGCAATATCTCCTCAACATAGGCAACGTTAACTTCGCCGGGGCGATGGCTGACCAGGCTAATACCC TGAACGGAGCTATCGCTAGTGCGGAGGACTCATGGTCCCAGTTGAAGATGACTCTCGCTACTAGTCTGGATGTAGGGTCGCTGGCGGAAC CTTTACGTTATATTGACGACCTGATACAGGAGATAAACGCTCAGGTCGCATCCGGTGAGTTCGTAGCCGAGATGCGGATGTGGGGTGACAT GGCATCCGATGTTGGGGGTGCGATAGAGGCTTCATTCGACGCGGCGTTTGGGATGGTCGCAGACGCGTTAAACGCTTTGAACTCCGCCTG GACTTACACCAGTGAAAGCATTACCGGAAGCGGAGAGGAGACCGCATCTACGATAGCTGAGTCAGCGGCGGATGCGCTGGACTTCATTGC CCAGGAATTTACGGCAATGGAGCGGTTTTTTGAAGATATGGTTAAAGGTGCTCAGGATGCAGGACGTCTTGTAAAGGCCGCATTGACGCCG GGAGAGTCGGTAGCTGAGGCCAAGAACCTTAACTTCCAGTTAGCGCTTGCTATGGATACTCAAAGAGCTGTGACTGACCTGACACGTAAAA GTTTCCGTGAACAGGTAGAAGCTCAGGAAGACCTTATCGCATTGAAGCGTGCGGCTTACGACATCGATAAAGAAGCGGCCAAGGCCGAGG GTCTTGGTAAGTTTAAAGTGTCGGGTAAAGATAACGGTTCTACAGGAGATTCCGCAGACAAGGCCGCTAAGAAATCCGTCGACGCATTCGA ACGCCAGAAGAAAGCCGCTGAGGATTTCTATTACCAGTCAATCCACCTTAACGATGACGTATTCCAGAAGATACAAGCTAACCAAGAAGAG CAACTTACTAAGCTACAGGAGTTCTTCAGCAACCGTCTCCTTAGTGACCAGCAATACGAAACCGCTAAGACGCAGATTATGCTCGAGGCGG ATACGGCCCGCCAGGCTGAGTTAGATAAACGCGAGAAAGAGCGCCTGGAAAAACAATTCTCCGCAGATGCCTACGTCGCTCAGATGCAGG CCCTTGCCGAAGGGGAATTCGCCGAGTTAGACCGTCAGTACGAGGTCAAGCTACAAAAACTTAACGACTTTCATGCGCAGGGTTTAATCGC GGAAGAAACATACCAGCAGACCCTAAACGCAATGAATGATACTTACGCATTAGACCGAGCGAAGGCGACAGGCACCGCATTTGGTAATATG GCTAGTAACATCGGGGCCGCGCTAGGAGAGGCTTCCACGGCATACAAAGCATTTGCCATCGCACAGGCGACAATCGCTACGTACACGTCA GCAGTAGAAGCGTATAAATCAACAGCAGCCATACCGGTAGTCGGCCCGTATCTGGCGCCTGTTGCTGCGGCTGCTGCGGTGGCGGCTGG TTTAGCAAACGTAGGTAAGATTCGCTCCGCCCGAGAACAGGGTGGTAACCTGGCCGCAGGGCAGATATCCACTATTGCAGAACGTGGTAA ACCAGAAGTAATCATGCCCGCTAGCGCCTCCCGTGTCCGGACGGCGGAGCAGATGCGACAGATTATGGGCGAGAACGACGCTAAATCCG GCGGGGATAATGTTACTATTGTGAACAATACCACTGGAAGAATTGATTCTGCTGCAACAGAACGCGACGATGAAGGCCGGTTGCGTATTAT AATCAGTGAAACCGTAAGTTCAGCGTTGCTGGATAGTAACAGCGCCATTTCTAAATCACGTCGCGCTACACGCGGCCAACCAGGATATTGA
40	ATGGCGTTACAACCATATAAGGGCGCGATGACCGCGCAATTTTACGTTCTTGAGACGACGCCGGGTGTGACGCCCGATAATCCGGTATGG CAGCCGCTCCGTAACACCGGCGGCATTCCAGCCGTAACGCGCGACGCCCTCATCTCTAACGAGCTGGACGGCAGCCGCGAAACATCATC TATCCGCACCGGTAACCGTCAGGTAACTGGTGAATACGCTATCGAACTAAGCGCGACAAGCCAGGATGAGTTGCTTGCGGGCGCAATGAC CAGTTCCTGGGTAGCAGGTTCCACTAAATCTGGAATCAGCGTTACCGTAGACCCAGCAGTGAAAACTTTCACACGCGCTACTGGTAGCTTT GTAACCGATGGCGTTGAAGTAGGCGACCTGGTGCAATTCGACGGCCTGTCTGGCAATAATGACAAAGCTTTCCTCGTTACCGCAGTAACTG CTACGGTTGTAACCGGTGCCGGTATCCAGCATACCCTTACCGCCGAATCAGACGCCCAAGCCGATTTGCGTATCGCAGATAAACTTGAAA CTGGTAACTTGTGTAAAACCTATTCAATCTTGACATGGTTGAAAGGTAAATGCGGAAACCCGGATTCATACATCATAACCCGCGGAGTCGAG TTTACCGGGTTCACTATCGAACAGGCAGTTAACGCGATGGTGACCGGCTCTTTCCCGTTCATTGGCTTGAATCAGGAAATCCTACAAACAC CGCCGAGCGGTTCAGATTTCACGACCAATTTTAGCGCCCGCCCGTTTGCATCGGTTGATGTATCCGCTTATGACGGAGCCGCGCCGCTTA AACTTATCGACACGTTCACCATTACTAACGACAACAGCGCGTCCGCACAGTTCGAGTTAGGAAATAACAGCGTGGCATTTGTCGAACGTGG CCGCGCGGCTAACACCTTCTCGTTGGCGGGTAAGCTGTACGACATGACGTTATTGAATAAATTCCTGAACGAAACGCAAATGGAGGTATCT TCTGTTCTGAACGGCCCAGACGGTGCCATGAGTTTCACCTTAAAACGCGCTTCGTTGACATCAGCAACTCCGGAAATCGGTGGTCCCGAAT CTGTCACCCTTTCTCTTGAGGGACAGGCAACCGGTAACCAGTTCCAGTCTTCAATTGTTATCCAGCGCATTAAGTACACCTAA
47	ATGGTTGATGTAATTAAACGTCGTATTGTTGGGGTATCTGATGACAGCCCGCAGGATGGGCAGGTTGAGATTGATATGGAAAACGTGACGC CGTTGCGTTTCTCTACAGGTCTCGATGATACCACCGCGGTAACAGCCGGGCAGGCTATCACCCTGACTGTAGCGCTTGCCGACGGAATGG GCCCTAAAACGGTTCAATGGTATAAGGATAATAACGCCATCTCCGGCGCAACCGGTTTGACTTATACGAAGGCCAACTCCGCCGCAGCGG ATTCTGGCACCTATAAAGTCGTAGCACATGACGGGTACGGTAACATTATCTCAGATAGCACAGTAGTAACCGTAAGTTAA
54	ATGGGCTTTTTCAAAGTTAAAGATGTGCCTTCACGACGTGTAGTTCAGTACTCCCGTGTGTCCGGCGCGGGCGAAGGTGTTGTGTACATTA AAGATGAATCTGTTCTTGGTGAGTCGGTAGATGAAATGCCGTTCGCTGATAAGACCGGACTGGCAGCAATCCCTGACGGTATCCTGTACGA AGTGCCGTATCTGGACGGAGCAGGTGATGTGTACTTCGACACACAGCCAGCAGATGTAGAATTGAAAGACGGTTCCGCTAAACTAACCGTA GTCGTGAAAGGTGGCAAAGCGCCCTACGATTTGCAATGGTTTAAAAATGGTAAAGAGGTAATCAACGTCCCTTATGTTGAAGGGGAACTAA CTGTTAAAGACCCGGGGGAGTATTTCGTCAGGGCGGTAGATGCTGACGGGATATCGGTGGTAAGTAAAGCGGCTAAGGTCTCAGAACCTA AGTAA

Table 20- Nucleotide sequence from T4 genes

Genes	T4
12	ATGAGTAATAATACATATCAACACGTTTCTAATGAATCTCGTTATGTAAAATTTGATCCTACCGATACGAATTTTCCACCGGAGATTAC TGATGTTCACGCTGCTATAGCAGCCATTTCTCCTGCTGGAGTAAATGGAGTTCCTGATGCATCGTCAACAACAAAGGGAATTCTATTT ATTCCCACTGAACAGGAAGTTATAGATGGAACTAATAATACCAAAGCAGTTACACCAGCAACGTTGGCAACAAGATTATCTTATCCAA ATGCAACTGAAACTGTTTACGGATTAACAAGATATTCAACCAATGATGAAGCCATTGCCGGAGTTAATAATGAATCTTCTATAACTCC AGCTAAATTTACTGTCGCCCTTAATAATGCGTTTGAAACGCGAGTTTCAACTGAATCCTCAAATGGTGTTATTAAAATTTCATCTCTAC CGCAAGCATTAGCTGGTGCAGATGATACTACTGCAATGACTCCATTAAAAACACAGCAGTTAGCTATTAAATTAATTGCGCAAATTGC TCCTTCTGAAACCACAGCTACCGAATCGGACCAAGGTGTTGTTCAATTAGCAACAGTAGCGCAGGTTCGTCAGGGAACTTTAAGAGA AGGCTATGCAATTTCTCCTTATACGTTTATGAATTCATCTTCTACTGAAGAATATAAAGGCGTAATTAAATTAGGAACACAATCAGAAG TTAACTCGAATAATGCTTCTGTTGCGGTTACTGGCGCAACTCTTAATGGTCGTGGTTCTACGACGTCAATGAGAGGCGTAGTTAAATT AACTACAACCGCCGGTTCACAGAGTGGAGGCGATGCTTCATCAGCCTTAGCTTGGAATGCTGACGTTATCCAGCAAAGAGGTGGTC AAATTATCTATGGAACACTCCGCATTGAAGACACATTTACAATAGCTAATGGTGGAGCAAATATTACGGGTACCGTCAGAATGACTGG CGGTTATATTCAAGGTAACCGCATCGTAACACAAAATGAAATTGATAGAACTATTCCTGTCGGAGCTATTATGATGTGGGCCGCTGAT AGTCTTCCTAGTGATGCTTGGCGCTTCTGCCATGGTGGAACTGTTTCAGCGTCAGATTGTCCATTATATGCTTCTAGAATTGGAACAA GATATGGCGGAAACCCATCAAATCCTGGATTGCCTGACATGCGTGGTCTTTTTGTTCGTGGTTCTGGTCGTGGTTCTCACTTAACAA ATCCAAATGTTAATGGTAATGACCAATTTGGTAAACCTAGATTAGGTGTAGGTTGTACCGGTGGATATGTTGGTGAAGTACAGATACA ACAGATGTCTTATCATAAACATGCTGGTGGATTTGGTGAGCATGATGATCTGGGGGCATTCGGTAATACCCGTAGATCAAATTTTGTT GGTACACGTAAAGGACTTGACTGGGATAACCGTTCATACTTCACCAATGACGGATATGAAATTGACCCAGAATCACAACGAAATTCC AAATATACATTAAATCGTCCTGAATTAATTGGAAATGAAACACGTCCATGGAACATTTCTTTAAACTACATAATTAAGGTAAAAGAATG A
37	ATGGCTACTTTAAAACAAATACAATTTAAAAGAAGCAAAATCGCAGGAACACGTCCTGCTGCTTCAGTATTAGCCGAAGGTGAATTGG CTATAAACTTAAAAGATAGAACAATTTTTACTAAAGATGATTCAGGAAATATCATCGATCTAGGTTTTGCTAAAGGCGGGCAAGTTGAT GGCAACGTTACTATTAACGGACTTTTGAGATTAAATGGCGATTATGTACAAACAGGTGGAATGACTGTAAACGGACCCATTGGTTCTA CTGATGGCGTCACTGGAAAAATTTTCAGATCTACACAGGGTTCATTTTATGCAAGAGCAACAAACGATACTTCAAATGCCCATTTATG GTTTGAAAATGCCGATGGCACTGAACGTGGCGTTATATATGCTCGCCCTCAAACTACAACTGACGGTGAAATACGCCTTAGGGTTAG ACAAGGAACAGGAAGCACTGCCAACAGTGAATTCTATTTCCGCTCTATAAATGGAGGCGAATTTCAGGCTAACCGTATTTTAGCATC AGATTCGTTAGTAACAAAACGCATTGCGGTTGATACCGTTATTCATGATGCCAAAGCATTTGGACAATATGATTCTCACTCTTTGGTTA ATTATGTTTATCCTGGAACCGGTGAAACAAATGGTGTAAACTATCTTCGTAAAGTTCGCGCTAAGTCCGGTGGTACAATTTATCATGA AATTGTTACTGCACAAACAGGCCTGGCTGATGAAGTTTCTTGGTGGTCTGGTGATACACCAGTATTTAAACTATACGGTATTCGTGAC GATGGCAGAATGATTATCCGTAATAGCCTTGCATTAGGTACATTCACTACAAATTTCCCGTCTAGTGATTATGGCAACGTCGGTGTAA TGGGCGATAAGTATCTTGTTCTCGGCGACACTGTAACTGGCTTGTCATACAAAAAAACTGGTGTATTTGATCTAGTTGGCGGTGGATA TTCTGTTGCTTCTATTACTCCTGACAGTTTCCGTAGTACTCGTAAAGGTATATTTGGTCGTTCTGAGGACCAAGGCGCAACTTGGATA ATGCCTGGTACAAATGCTGCTCTCTTGTCTGTTCAAACACAAGCTGATAATAACAATGCTGGAGACGGACAAACCCATATCGGGTAC AATGCTGGCGGTAAAATGAACCACTATTTCCGTGGTACAGGTCAGATGAATATCAATACCCAACAAGGTATGGAAATTAACCCGGGT ATTTTGAAATTGGTAACTGGCTCTAATAATGTACAATTTTACGCTGACGGAACTATTTCTTCCATTCAACCTATTAAATTAGATAACGA GATATTTTTAACTAAATCTAATAATACTGCGGGTCTTAAATTTGGAGCTCCTAGCCAAGTTGATGGCACAAGGACTATCCAATGGAAC GGTGGTACTCGCGAAGGACAGAATAAAAACTATGTGATTATTAAAGCATGGGGTAACTCATTTAATGCCACTGGTGATAGATCTCGC GAAACGGTTTTCCAAGTATCAGATAGTCAAGGATATTATTTTTATGCTCATCGTAAAGCTCCAACCGGCGACGAAACTATTGGACGTA TTGAAGCTCAATTTGCTGGGGATGTTTATGCTAAAGGTATTATTGCCAACGGAAATTTTAGAGTTGTTGGGTCAAGCGCTTTAGCCGG CAATGTTACTATGTCTAACGGTTTGTTTGTCCAAGGTGGTTCTTCTATTACTGGACAAGTTAAAATTGGCGGAACAGCAAACGCACTG AGAATTTGGAACGCTGAATATGGTGCTATTTTCCGTCGTTCGGAAAGTAACTTTTATATTATTCCAACCAATCAAAATGAAGGAGAAAG TGGAGACATTCACAGCTCTTTGAGACCTGTGAGAATAGGATTAAACGATGGCATGGTTGGGTTAGGAAGAGATTCTTTTATAGTAGAT CAAAATAATGCTTTAACTACGATAAACAGTAACTCTCGCATTAATGCCAACTTTAGAATGCAATTGGGGCAGTCGGCATACATTGATG CAGAATGTACTGATGCTGTTCGCCCGGCGGGTGCAGGTTCATTTGCTTCCCAGAATAATGAAGACGTCCGTGCGCCGTTCTATATGA ATATTGATAGAACTGATGCTAGTGCATATGTTCCTATTTTGAAACAACGTTATGTTCAAGGCAATGGCTGCTATTCATTAGGGACTTTA ATTAATAATGGTAATTTCCGAGTTCATTACCATGGCGGCGGAGATAACGGTTCTACAGGTCCACAGACTGCTGATTTTGGATGGGAAT TTATTAAAAACGGTGATTTTATTTCACCTCGCGATTTAATAGCAGGCAAAGTCAGATTTGATAGAACTGGTAATATCACTGGTGGTTCT GGTAATTTTGCTAACTTAAACAGTACAATTGAATCACTTAAAACTGATATCATGTCGAGTTACCCAATTGGTGCTCCGATTCCTTGGC CGAGTGATTCAGTTCCTGCTGGATTTGCTTTGATGGAAGGTCAGACCTTTGATAAGTCCGCATATCCAAAGTTAGCTGTTGCATATCC TAGCGGTGTTATTCCAGATATGCGCGGGCAAACTATCAAGGGTAAACCAAGTGGTCGTGCTGTTTTGAGCGCTGAGGCAGATGGTGT TAAGGCTCATAGCCATAGTGCATCGGCTTCAAGTACTGACTTAGGTACTAAAACCACATCAAGCTTTGACTATGGTACGAAGGGAAC TAACAGTACGGGTGGACACACTCACTCTGGTAGTGGTTCTACTAGCACAAATGGTGAGCACAGCCACTACATCGAGGCATGGAATG GTACTGGTGTAGGTGGTAATAAGATGTCATCATATGCCATATCATACAGGGCGGGTGGGAGTAACACTAATGCAGCAGGGAACCACA GTCACACTTTCTCTTTTGGGACTAGCAGTGCTGGCGACCATTCCCACTCTGTAGGTATTGGTGCTCATACCCACACGGTAGCAATTG GATCACATGGTCATACTATCACTGTAAATAGTACAGGTAATACAGAAAACACGGTTAAAAACATTGCTTTTAACTATATCGTTCGTTTA GCATAA
57	ATGTCTGAACAAACTGTTGAACAAAAACTGTCTGCTGAAATCGTAACTCTGAAATCTCGCATTCTTGATACGCAGGATCAAGCTGCTC GTCTGATGGAAGAATCCAAAATTCTGCAAGGAACTTTGGCTGAAATTGCTCGTGCAGTAGGTATCACTGGCGATACTATCAAAGTTGA AGAAATCGTTGAAGCTGTCAAAAATCTTACTGCTGAATCTGCAGATGAAGCAAAAGATGAAGAATGA

38	ATGAAAATATATCATTATTATTTTGACACTAAAGAATTTTACAAAGAAGAAAATTACAAACCGGTTAAAGGCCTCGGTCTTCCTGCTCA TTCAACAATTAAAAAACCTTTAGAACCTAAAGAAGGATACGCGGTTGTATTTGATGAACGTACTCAGGATTGGATTTATGAAGAAGAC CATCGCGGAAAACGCGCATGGACTTTTAATAAAGAAGAAATTTTTATAAGTGACATTGGAAGCCCGGTTGGTATAACTTTCGATGAGC CCGGCGAATTTGATATATGGACTGATGACGGTTGGAAAGAAGACGAAACATATAAGCGAGTTTTAATTCGTAATAGAAAAATTGAAGA ATTATATAAAGAGTTCCAAGTTTTAAATAATATGATTGAAGCTTCTGTCGCCAATAAAAAGGAAAAATTCTATTATAAAAACCTTAAGCG GTTCTTTGCTCTTTTAGAAAAGCATGAGCATTTAGGTGGTGAATTCCCTTCATGGCCTGAAAAAGAACAGAAGCCTTGGTATAAGCGT TTATTCAAGCATTACGTATAA
37_1380	GATGGCACAAGGACTATCCAATGGAACGGTGGTACTCGCGAAGGACAGAATAAAAACTATGTGATTATTAAAGCATGGGGTAACTCA TTTAATGCCACTGGTGATAGATCTCGCGAAACGGTTTTCCAAGTATCAGATAGTCAAGGATATTATTTTTATGCTCATCGTAAAGCTC CAACCGGCGACGAAACTATTGGACGTATTGAAGCTCAATTTGCTGGGGATGTTTATGCTAAAGGTATTATTGCCAACGGAAATTTTAG AGTTGTTGGGTCAAGCGCTTTAGCCGGCAATGTTACTATGTCTAACGGTTTGTTTGTCCAAGGTGGTTCTTCTATTACTGGACAAGTT AAAATTGGCGGAACAGCAAACGCACTGAGAATTTGGAACGCTGAATATGGTGCTATTTTCCGTCGTTCGGAAAGTAACTTTTATATTA TTCCAACCAATCAAAATGAAGGAGAAAGTGGAGACATTCACAGCTCTTTGAGACCTGTGAGAATAGGATTAAACGATGGCATGGTTG GGTTAGGAAGAGATTCTTTTATAGTAGATCAAAATAATGCTTTAACTACGATAAACAGTAACTCTCGCATTAATGCCAACTTTAGAATG CAATTGGGGCAGTCGGCATACATTGATGCAGAATGTACTGATGCTGTTCGCCCGGCGGGTGCAGGTTCATTTGCTTCCCAGAATAAT GAAGACGTCCGTGCGCCGTTCTATATGAATATTGATAGAACTGATGCTAGTGCATATGTTCCTATTTTGAAACAACGTTATGTTCAAG GCAATGGCTGCTATTCATTAGGGACTTTAATTAATAATGGTAATTTCCGAGTTCATTACCATGGCGGCGGAGATAACGGTTCTACAGG TCCACAGACTGCTGATTTTGGATGGGAATTTATTAAAAACGGTGATTTTATTTCACCTCGCGATTTAATAGCAGGCAAAGTCAGATTTG ATAGAACTGGTAATATCACTGGTGGTTCTGGTAATTTTGCTAACTTAAACAGTACAATTGAATCACTTAAAACTGATATCATGTCGAGT TACCCAATTGGTGCTCCGATTCCTTGGCCGAGTGATTCAGTTCCTGCTGGATTTGCTTTGATGGAAGGTCAGACCTTTGATAAGTCC GCATATCCAAAGTTAGCTGTTGCATATCCTAGCGGTGTTATTCCAGATATGCGCGGGCAAACTATCAAGGGTAAACCAAGTGGTCGT GCTGTTTTGAGCGCTGAGGCAGATGGTGTTAAGGCTCATAGCCATAGTGCATCGGCTTCAAGTACTGACTTAGGTACTAAAACCACA TCAAGCTTTGACTATGGTACGAAGGGAACTAACAGTACGGGTGGACACACTCACTCTGGTAGTGGTTCTACTAGCACAAATGGTGAG CACAGCCACTACATCGAGGCATGGAATGGTACTGGTGTAGGTGGTAATAAGATGTCATCATATGCCATATCATACAGGGCGGGTGG GAGTAACACTAATGCAGCAGGGAACCACAGTCACACTTTCTCTTTTGGGACTAGCAGTGCTGGCGACCATTCCCACTCTGTAGGTAT TGGTGCTCATACCCACACGGTAGCAATTGGATCACATGGTCATACTATCACTGTAAATAGTACAGGTAATACAGAAAACACGGTTAAA AACATTGCTTTTAACTATATCGTTCGTTTAGCATAA
37@726	GGTAATATCACTGGTGGTTCTGGTAATTTTGCTAACTTAAACAGTACAATTGAATCACTTAAAACTGATATCATGTCGAGTTACCCAAT TGGTGCTCCGATTCCTTGGCCGAGTGATTCAGTTCCTGCTGGATTTGCTTTGATGGAAGGTCAGACCTTTGATAAGTCCGCATATCC AAAGTTAGCTGTTGCATATCCTAGCGGTGTTATTCCAGATATGCGCGGGCAAACTATCAAGGGTAAACCAAGTGGTCGTGCTGTTTT GAGCGCTGAGGCAGATGGTGTTAAGGCTCATAGCCATAGTGCATCGGCTTCAAGTACTGACTTAGGTACTAAAACCACATCAAGCTT TGACTATGGTACGAAGGGAACTAACAGTACGGGTGGACACACTCACTCTGGTAGTGGTTCTACTAGCACAAATGGTGAGCACAGCC ACTACATCGAGGCATGGAATGGTACTGGTGTAGGTGGTAATAAGATGTCATCATATGCCATATCATACAGGGCGGGTGGGAGTAACA CTAATGCAGCAGGGAACCACAGTCACACTTTCTCTTTTGGGACTAGCAGTGCTGGCGACCATTCCCACTCTGTAGGTATTGGTGCTC ATACCCACACGGTAGCAATTGGATCACATGGTCATACTATCACTGTAAATAGTACAGGTAATACAGAAAACACGGTTAAAAACATTGC TTTTAACTATATCGTTCGTTTAGCATAA

Table 21- Aminoacidic sequence from T4 genes

Protein	Amino acid sequence
gp27	MSSGCGDVLSLNDLQIAKKHQIFEAEVITGKQGGVAGGADIDYATNQVTGQTQKTLPAVLRDAGFSPASFNFTTGGTLGVNDADKAVL WPKEDGGDGNYYAWRGPLPKVIPAASTPLTTGGISDSAWVAFGDITFRAEADKKFKYSVKLSDFTTLQQLADAAVDSVLIDRDYNFSN NETVNFGGKTLTIDCKAKFIGDGNLVFTQLGRGSVVVGAYMESVTTPWVIKPWTDDNQWITDPAAIVATLKQSKTDGYQPTVNDYAKF PGIESLLPPEAKDQNISSVLEIRECTGVEVHRASGLMACFLFRGCHFCKMVDADNPSGGKDGVITFENLSGDWGKGNYVIGGRTSYG SVSSAQFLRNNGGFARDGGVIGFTSYRAGESGVKTWQGTVGSTTSRNYNLQFRDSAVLYPVWDGFDLGADTDMNPEDDRPGDFPIS QYPVHMLPLNHLIDNLFVRGSLGVGFGMDGQGLYVSNITVEDCAGSGAYILAHETVFTNIAIIDTNTKNFPANQIYISGACRVNGLRLVG IRSTSEQGLTIDAPNSTVSGITGFVDPSRINVANLMEEGLGNSRINSFNNGSAALRFRIHKLSKTLDSGSVYSHLNGGPGSGSAWTEIT AIAGSLPDAVSLKINRGDYRAVEIPVAMSVLPDNAVRDNGSISLYLEGDSLKALVKRADGSYTRLTLA
gp28	MALVIHYTRNEDGTFDVKRYRDNPMNFVVNHVPDGVPVRVFIDEIGEDNDVTEDFEALKENATFHIVESAGGGAIKGVMKIFSVILKPL AKLLSPSVKGASSNLANSQADSPNNSLTDRNNKARPYERSYDICGTVQTIPNNLMSTYKVFNAAGKIVEYGYYDAGRGYLDIHPEGIT DGDTRVSDITGTSVAVYAPYTSPNNTSTPQVMVGDPIEQGLYITVESNEVDGVVLKAPNGLGISFSYMSGYPSLSGNIGTIYDPTGGSDF SGVLVPNDTFSLVSAWTNTDVDLSGGGYQVVSVSEGTVTFIVPGGLIGRWQEIRPGSFFRGDGEASLQPDNAYEKTLTDWVSINRTEV ERIVANIAAANGMYKDNGKSKTLASVTAEIQYQLLDENSTPYGPIYTAQGTVSGRTPDYNGVTIYADLPVVSRVRVRARRVTDLDFNFEG SVVDEITYVNLYGQTRDNTPHYGNRTTVHSMRKQTPRAAEVKQPQLRMIATEMVYKYLGNGVFEDTMTPNTQAVQSLIRLARDPDVG GLNLTVRNMDKLLAVQNEVEAYFGDKQAGEFCYTFDDYKTTMQDIVSTIADAIFCTPYRRGADILLDFERPRMGPEMVFTHRSKAGTS EKWTRTFNDSQVFDSLKFSYIDPKTNVKETITIPETGGLKTETYDSKGIRNYKQAFWAANRRHQKNILKKISVSFTATEEGIFALPNRAV SVVKGSRMSTYDGYVTAVNGLTVELSQPVKFTSGDDHYLVLKLRDGGVQSVRVVPGAHDRQVIMTSVPQEAIYTGNSALKTEFSFGNE ARHNAQMILVSTVDPGDDRTVKITGFNYDKDFYKFDNVPPFGRAFSSGFDNGFN
gp34-32	MADVASLVVKVTEQGAKATSDRLDNLSKSAKVAGAAVTGLAAVVAATAYKAAQELVESQRQLDKMSASLKTLTGSTQGAKQALSILQ DFARDTPYGLEQAVEGFRKLVALGLTPSEEALRSYGNTASAMGKDLNQMIEAVADASTFEFERLKEFGIKAKQNQSDIEFTFQGTTTV VKKNAADIEQYLLNIGNVNFAGAMADQANTLNGAIASAEDSWSQLKMTLATSLDVGSLAEPLRYIDDLIQEINAQVASGEFVAEMRMW GDMASDVGGAIEASFDAAFGMVADALNALNSAWTYTSESITGSGEETASTIAESAADALDFIAQEFTAMERFFEDMVKGAQDAGRLVK AALTPGESVAEAKNLNFQLALAMDTQRAVTDLTRKSFREQVEAQEDLIALKRAAYDIDKEAAKAEGLGKFKVSGKDNGSTGDSADKA AKKSVDAFERQKKAAEDFYYQSIHLNDDVFQKIQANQEEQLTKLQEFFSNRLLSDQQYETAKTQIMLEADTARQAELDKREKERLEK QFSADAYVAQMQALAEGEFAELDRQYEVKLQKLNDFHAQGLIAEETYQQTLNAMNDTYALDRAKATGTAFGNMASNIGAALGEASTA YKAFAIAQATIATYTSAVEAYKSTAAIPVVGPYLAPVAAAAAVAAGLANVGKIRSAREQGGNLAAGQISTIAERGKPEVIMPASASRVRTAE QMRQIMGENDAKSGGDNVTIVNNTTGRIDSAATERDDEGRLRIIISETVSSALLDSNSAISKSRRATRGQPGY MVPAWRNYQPVTDQPKGITMKLSDFYYEAEAEKGARMPIPLKDGTDSGEWLNVVSPEADVAVKAMRAFTLAYRAAVGKLKPLRDKC EEQKDFSEYNLKMEDAAGDLNRQLALELVNGWSLDDEFTKENLKTLLAQYKRLAEHVVVFHHEQLRQLQEKMFQFARWNFITRHE RRKFDSIADGHKAALIAMGVIKDAGETTQDAGPECPPELLTTFEKYRDAKFTRRVDDDGVKLYPREQLSWSDLVAYSTISGQNIGMFE SEIIMGLDAIFEGRNDG
gp40	MALQPYKGAMTAQFYVLETTPGVTPDNPVWQPLRNTGGIPAVTRDALISNELDGSRETSSIRTGNRQVTGEYAIELSATSQDELLAGA MTSSWVAGSTKSGISVTVDPAVKTFTRATGSFVTDGVEVGDLVQFDGLSGNNDKAFLVTAVTATVVTGAGIQHTLTAESDAQADLRIAD KLETGNLCKTYSILTWLKGKCGNPDSYIITRGVEFTGFTIEQAVNAMVTGSFPFIGLNQEILQTPPSGSDFTTNFSARPFASVDVSAYDG AAPLKLIDTFTITNDNSASAQFELGNNSVAFVERGRAANTFSLAGKLYDMTLLNKFLNETQMEVSSVLNGPDGAMSFTLKRASLTSAT PEIGGPESVTLSLEGQATGNQFQSSIVIQRIKYT
gp47	MVDVIKRRIVGVSDDSPQDGQVEIDMENVTPLRFSTGLDDTTAVTAGQAITLTVALADGMGPKTVQWYKDNNAISGATGLTYTKANSA AADSGTYKVVAHDGYGNIISDSTVVTVS
gp54	MGFFKVKDVPSRRVVQYSRVSGAGEGVVYIKDESVLGESVDEMPFADKTGLAAIPDGILYEVPYLDGAGDVYFDTQPADVELKDGSAK LTVVVKGGKAPYDLQWFKNGKEVINVPYVEGELTVKDPGEYFVRAVDADGISVVSKAAKVSEPK

Table 22- Aminoacidic sequence from T4 genes

T4	Amino acid sequence
gp12	MSNNTYQHVSNESRYVKFDPTDTNFPPEITDVHAAIAAISPAGVNGVPDASSTTKGILFIPTEQEVIDGTNNTKAV TPATLATRLSYPNATETVYGLTRYSTNDEAIAGVNNESSITPAKFTVALNNAFETRVSTESSNGVIKISSLPQALAG ADDTTAMTPLKTQQLAIKLIAQIAPSETTATESDQGVVQLATVAQVRQGTLREGYAISPYTFMNSSSTEEYKGVIK LGTQSEVNSNNASVAVTGATLNGRGSTTSMRGVVKLTTTAGSQSGGDASSALAWNADVIQQRGGQIIYGTLRIE DTFTIANGGANITGTVRMTGGYIQGNRIVTQNEIDRTIPVGAIMMWAADSLPSDAWRFCHGGTVSASDCPLYAS RIGTRYGGNPSNPGLPDMRGLFVRGSGRGSHLTNPNVNGNDQFGKPRLGVGCTGGYVGEVQIQQMSYHKHA GGFGEHDDLGAFGNTRRSNFVGTRKGLDWDNRSYFTNDGYEIDPESQRNSKYTLNRPELIGNETRPWNISLNY IIKVKE
gp37	MATLKQIQFKRSKIAGTRPAASVLAEGELAINLKDRTIFTKDDSGNIIDLGFAKGGQVDGNVTINGLLRLNGDYVQ TGGMTVNGPIGSTDGVTGKIFRSTQGSFYARATNDTSNAHLWFENADGTERGVIYARPQTTTDGEIRLRVRQGT GSTANSEFYFRSINGGEFQANRILASDSLVTKRIAVDTVIHDAKAFGQYDSHSLVNYVYPGTGETNGVNYLRKVR AKSGGTIYHEIVTAQTGLADEVSWWSGDTPVFKLYGIRDDGRMIIRNSLALGTFTTNFPSSDYGNVGVMGDKYLV LGDTVTGLSYKKTGVFDLVGGGYSVASITPDSFRSTRKGIFGRSEDQGATWIMPGTNAALLSVQTQADNNNAGD GQTHIGYNAGGKMNHYFRGTGQMNINTQQGMEINPGILKLVTGSNNVQFYADGTISSIQPIKLDNEIFLTKSNN TAGLKFGAPSQVDGTRTIQWNGGTREGQNKNYVIIKAWGNSFNATGDRSRETVFQVSDSQGYYFYAHRKAPTG DETIGRIEAQFAGDVYAKGIIANGNFRVVGSSALAGNVTMSNGLFVQGGSSITGQVKIGGTANALRIWNAEYGAIF RRSESNFYIIPTNQNEGESGDIHSSLRPVRIGLNDGMVGLGRDSFIVDQNNALTTINSNSRINANFRMQLGQSAY IDAECTDAVRPAGAGSFASQNNEDVRAPFYMNIDRTDASAYVPILKQRYVQGNGCYSLGTLINNGNFRVHYHGG GDNGSTGPQTADFGWEFIKNGDFISPRDLIAGKVRFDRTGNITGGSGNFANLNSTIESLKTDIMSSYPIGAPIPW PSDSVPAGFALMEGQTFDKSAYPKLAVAYPSGVIPDMRGQTIKGKPSGRAVLSAEADGVKAHSHSASASSTDL GTKTTSSFDYGTKGTNSTGGHTHSGSGSTSTNGEHSHYIEAWNGTGVGGNKMSSYAISYRAGGSNTNAAGNH SHTFSFGTSSAGDHSHSVGIGAHTHTVAIGSHGHTITVNSTGNTENTVKNIAFNYIVRLA
gp37_1380	DGTRTIQWNGGTREGQNKNYVIIKAWGNSFNATGDRSRETVFQVSDSQGYYFYAHRKAPTGDETIGRIEAQFAG DVYAKGIIANGNFRVVGSSALAGNVTMSNGLFVQGGSSITGQVKIGGTANALRIWNAEYGAIFRRSESNFYIIPTN QNEGESGDIHSSLRPVRIGLNDGMVGLGRDSFIVDQNNALTTINSNSRINANFRMQLGQSAYIDAECTDAVRPA GAGSFASQNNEDVRAPFYMNIDRTDASAYVPILKQRYVQGNGCYSLGTLINNGNFRVHYHGGGDNGSTGPQTA DFGWEFIKNGDFISPRDLIAGKVRFDRTGNITGGSGNFANLNSTIESLKTDIMSSYPIGAPIPWPSDSVPAGFALM EGQTFDKSAYPKLAVAYPSGVIPDMRGQTIKGKPSGRAVLSAEADGVKAHSHSASASSTDLGTKTTSSFDYGTK GTNSTGGHTHSGSGSTSTNGEHSHYIEAWNGTGVGGNKMSSYAISYRAGGSNTNAAGNHSHTFSFGTSSAGD HSHSVGIGAHTHTVAIGSHGH TITVNSTGNTENTVKNIAFNYIVRLA
gp37@726	GNITGGSGNFANLNSTIESLKTDIMSSYPIGAPIPWPSDSVPAGFALMEGQTFDKSAYPKLAVAYPSGVIPDMRG QTIKGKPSGRAVLSAEADGVKAHSHSASASSTDLGTKTTSSFDYGTKGTNSTGGHTHSGSGSTSTNGEHSHYI EAWNGTGVGGNKMSSYAISYRAGGSNTNAAGNHSHTFSFGTSSAGDHSHSVGIGAHTHTVAIGSHGHTITVNS TGNTENTVKNIAFNYIVRLA
gp38	MKIYHYYFDTKEFYKEENYKPVKGLGLPAHSTIKKPLEPKEGYAVVFDERTQDWIYEEDHRGKRAWTFNKEEIFI SDIGSPVGITFDEPGEFDIWTDDGWKEDETYKRVLIRNRKIEELYKEFQVLNNMIEASVANKKEKFYYKNLKRFF ALLEKHEHLGGEFPSWPEKEQKPWYKRLFKHYV
gp57	MSEQTVEQKLSAEIVTLKSRILDTQDQAARLMEESKILQGTLAEIARAVGITGDTIKVEEIVEAVKNLTAESADEAK DEE

Annex IV: Transformation protocol by electroporation and heat shock

a) Electroporation

After the electro-competent cells thawing on ice (≈ aliquots of 100 μL), it was added 2-5 μL of ligation or construction, and gently mixed and incubated for 10 minutes on ice. Then the mixture was transferred for a 2-mm cuvette and electroporated at 1800 V, 25 μF and 200 Ω in Gene Pulser Xcell (Bio-Rad) electroporator. Thereafter, 400 μL of SOC medium was added to the couvete to ressuspend the cells and these ones were immediately transferred to a 1.5 mL Eppendorf. The cells were left to recover during 2 hours at 37 °C and 120 rpm. Finally, the suspension was spread into LB agar Petri dishes containing the appropriate antibiotic, and the plates were incubated at 37 °C overnight.

b) Heat Shock

The chemical competent cells (≈ 80 μL) were thawed on ice for 20 and 5-10 μL of the construction were added, and then were incubated for 20 minutes on ice. The heat shock was performed at 42 °C for 70 s in a PCH2 Dry Block Heating/Cooling Systems (Grant-bio). The suspension was incubated on ice for 5 minutes and 400 μL of sterile SOC medium was added.

The cells recovered for 2h at 37 °C and 120 rpm, and then spread into LB agar Petri dishes containing the appropriate antibiotic. The plates were incubated at 37 °C overnight.

Annex V: SDS-PAGE gels

To prepare acrylamide gels at 12% it was used a recipe for three gels with amounts and reagents that can be visualized in Table 23. The ingredients adding order was also followed per the table order.

Table 23- SDS-PAGE components and quantities.

Reagents	Stacking gel (4%) µL	Running gel (12%) µL
Water	3120	6956
1.5 M Tris HCl (pH 8.8)	-	4000
0.5 M Tris HCl (pH 6.8)	1250	-
Acrylamide/Bis-Acrylamide (40%)	500	4800
10% SDS	50	160
20% APS	20	56
TMED	6	28

Annex VI: Protein Calibration curve

Using a 2 mg/mL BSA stock solution, several solutions with different final BSA concentration in a range of 0.25-2 mg/mL of BSA were prepared. A volume of 25 μL of each BSA solution was added at 200 μL of BSA working reagent (WR) into microplate wells, and the microplate was incubated for 30 minutes at 37 °C. Finally, it was measured the absorbance of all samples in a spectrophotometer at 562 nm.

With the absorbance values obtained for each solution it was drawn a calibration curve of the absorbance at 562 nm in function of the BSA concentration in mg/mL the curve is represented in Figure with a regression equation of $O.D._{(562\ nm)} = (0.59 \pm 0.06)\ [BSA] + (0.12 \pm 0.04)$.

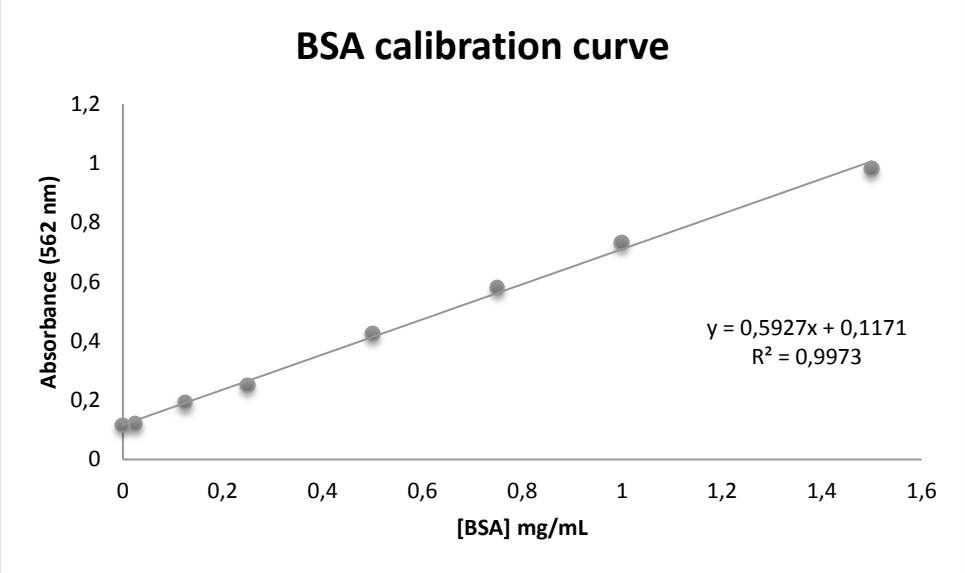


Figure 27- BSA calibration curve for protein concentration determination through the Pierce™ BCA Protein Assay.