OmpK antigenic peptide from *Vibrio alginolyticus* in recombinant *Escherichia coli* – production and secretion challenges

Filipa Catarina Camisão Saraiva Nunes

Thesis to obtain the Master of Science Degree in

Biotechnology

Supervisors:

Prof. Marília Clemente Velez Mateus

Prof. Sílvia Andreia Bento da Silva Sousa

Examination Committee

Chairperson: Prof. Leonilde de Fátima Morais Moreira

Supervisor: Prof. Sílvia Andreia Bento da Silva Sousa

Members of the committee: Prof. Joana Rita Rodrigues Feliciano

November 2021

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

During the realization of this project, I was able to count on the support and help of several people, and I must thank them.

I would like to start by thanking my advisors, Professor Marília Mateus and Doctor Sílvia Sousa, for all the kindness, patience and availability to help they showed throughout this project. Thank you for all the advice shared, for the demand and, above all, for the trust you have placed in me. I also want to thank Professor Jorge Leitão, for all his help and attention and for all the valuable ideas transmitted.

I leave here my thanks to my colleagues in the laboratory, for having received me in the best way, for their patience and understanding, for the knowledge and advice transmitted and for always showing themselves ready to help.

Finally, I leave my big thanks to my family and friends, who accompanied me at all times throughout this stage, always doing their best to understand and support me unconditionally. Thank you for all the patience and nice words shared with me. I will never forget everything you have done for me.

Abstract

Lpp is the Braun's lipoprotein responsible for the linkage between the peptidoglycan and the outer membrane to which structure defects can result in an improved protein leakage. In this work, an *E. coli* BL21 (DE3) strain with production of a *lpp* antisense sequence was constructed and the effect on cell permeability was evaluated. Additionally, overexpression of a OmpK 21-99 fragment of OmpK from *Vibrio alginolyticus* was optimized and the newly developed strain was used as a host to see the impact on peptide localization.

Lpp mRNA levels were evaluated by RT-PCR which allowed the determination of a decrease in the lpp transcript expression 2 hours after antisense induction, although 1.5 h later the antisense effect is lost possibly due to the antisense expression cells perishment. Monitorization of *E. coli* cells growth demonstrated a higher growth for the wild type culture comparing with the pMLBAD or the pSAS39 cultures, indicating the presence of the plasmid is the main cause for the growth disparity.

OmpK 21-99 fragment overexpression was verified at 30 ºC, although neither periplasmic localization nor extracellular medium secretion were confirmed, which might be related with problems in the detection procedure.

The evaluation of cells permeability under antisense effect, as well as the RT-PCR analysis showed very promising results, although certain aspects should be better studied. Overexpression of the OmpK fragment was verified but periplasmic and extracellular medium localization was not, meaning that the methodologies should be improved.

Keywords: fish vaccine, lpp antisense, OmpK, recombinant protein

Resumo

Lpp é a lipoproteína de Braun responsável pela ligação entre o peptidoglicano e a membrana externa à qual defeitos estruturais podem resultar numa melhoria da secreção de proteínas. Neste trabalho, uma estirpe de *E. coli* BL21 (DE3) com produção da sequência *antisense* lpp foi construída e o efeito na permeabilidade celular foi avaliado. Adicionalmente, otimização da sobreexpressão do fragmento OmpK 21-99 de *Vibrio alginolyticus* foi realizada, e a estirpe desenvolvida usada como hospedeira para observar o impacto na localização do péptido.

Os níveis de mRNA de lpp foram avaliados por RT-PCR, permitindo a observação de uma diminuição na expressão do transcrito 2h após a indução da expressão do *antisense*. No entanto, 1,5h depois ocorre uma perda do efeito do *antisense*, devido ao desaparecimento das células com expressão *antisense*. Monitorização do crescimento de *E. coli* demonstrou um maior crescimento na cultura *wild-type* comparativamente às culturas pMLBAD ou pSAS39, indicando que a presença do plasmídeo é a causa principal para as diferenças nos crescimentos.

Sobreexpressão do fragmento OmpK 21-99 foi verificada com sucesso a 30ºC, embora não se tenha comprovado a localização periplasmática do péptido nem a sua secreção para o meio extracelular, o que poderá estar relacionado com problemas no processo de deteção.

A avaliação da permeabilidade das células sob efeito do *antisense* e a análise de RT-PCR demonstraram resultados promissores, apesar de alguns aspetos ainda precisarem de ser melhor estudados. Sobreexpressão do fragmento OmpK foi confirmada ao contrário da localização periplasmática e citoplasmática, o que significa que as metodologias terão de ser melhoradas.

Palavras Chave: vacinação de peixes, lpp *antisense*, OmpK, proteínas recombinantes

Table of contents

List of Figures

List of tables

List of Abbreviations

A – absorbance
APS – Ammonium persulphate solution
ASO – Antisense oligonucleotides
bp – base pair
DNA – deoxyribonucleic acid
EDTA – Ethylenediamine tetraacetic acid
HPLC – High-performance liquid chromatography
IBs – Inclusion bodies
IPTG – Isopropyl β-D-1-thiogalactopyranoside
LB – Lennox broth
Lpp – Lipoprotein
LPS – Lipopolysaccharides
MWCO – Molecular weight cut-off
NacMur – *N*-acetylmuramic
NCBI – National Center for Biotechnology Information
ºC – degrees Celsius
OD – Optical density
OM – outer membrane
Omps – Outer membrane proteins
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
PG – Peptidoglycan
rpm – revolutions per minute
RT-PCR – Real time Polymerase Chain Reaction
SB – Super Broth
SDS – Sodium dodecyl sulfate
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE – Tris-Acetate-EDTA
TCM – Tris-Calcium-Magnesium
TEMED – Tetramethylethylenedi
Tris – tris(hidroximetil)aminometano

1. Introduction

1.1. Expression systems

The use of various organisms to produce recombinant proteins has revolutionized the field of biochemistry and biopharmaceutical and agricultural industry (Demain & Vaishnav, 2009). Some of the host systems that are available include bacteria, yeasts, filamentous fungi, unicellular algae and even mammalian cells. The choice of which host cell will be used to produce the protein of interest will depend on the type of protein to be produced, which will reflect on different molecular tools, equipment or reagents (Rosano & Ceccarelli, 2014). For example, if the heterologous recombinant protein needs to go through post-translational modifications (for example, glycosylation), in order to be functional, bacterial expression systems may not be suitable and alternative host systems have to be used (Terpe, 2006). Nevertheless, bacterial expression systems remain attractive due to their ability to grow rapidly and at high cell densities, at the expense of cheap substrates. Additionally, the fact that their genomes are well-characterized, the availability of a large number of cloning vectors and mutant host strains, also contributes to the interest surrounding these types of systems. For production of elevated levels of recombinant proteins, the gene encoding the protein needs to be cloned in a vector downstream of a well-characterized promoter, capable of being regulated (Terpe, 2006).

1.1.1. *Escherichia coli* as host

E. coli is a Gram-negative bacterium most commonly used for heterologous protein production, not only in laboratories but also for industrial and pharmaceutical production, where large scale systems are already well established (Kay Terpe, 2006) and about 40% of marketed recombinant therapeutics come from *E. coli* (Ni & Chen, 2009). This organism is very well-studied and the advantages of using it as a host organism are well known. In addition to the advantages mentioned above for microbial systems, the transformation with exogenous DNA is quite fast and easy (Pope & Kent, 1996). One disadvantage of using *E. coli* as host system for recombinant protein production is the accumulation of lipopolysaccharide (LPS), commonly referred to as endotoxins, which are dangerous for humans and other mammals. Proteins employed in therapeutics need to be extensively purified in order to become completely free of endotoxins (Petsch & Anspach, 2000).

Normally, overexpressed recombinant proteins accumulate either in the cytoplasm or in the periplasmic space, although the cytoplasm is the principal choice for protein production because of the higher yields reported. *E. coli* BL21 and K12 are the most frequently used strains, with the first being *lon* and *ompT* protease deficient. The *lon* protease is responsible for the degradation of many foreign proteins (Gottesman, 1996), whereas the *ompT* protease degrades extracellular proteins (Grodberg & Dunn, 1988). The absence of these genes from the BL21 genome allows for the expression of recombinant proteins, since it prevents the degradation of the newly synthetized proteins (Grodberg & Dunn, 1988).

1.1.1.1. Obstacles associated with recombinant protein production in *E. coli*

Gene overexpression in a foreign host might result in several unwanted problems, with the majority of them being related with the difference between codon usage of *E. coli* bacteria and the overexpressed protein (Kane, 1995). These differences can block the translation event from happening due to the demand for tRNAs that may be rare or absent in the expression host chosen. It can also result in translational stalling, premature termination of translation, translation frameshift and incorporation of the wrong amino acids. Modifying culture conditions, for example by lowering the temperature, or changing media composition are strategies that might help to alleviate some problems associated with codon-usage. Many strains of *E. coli* have been engineered to be able to supply additional tRNAs under control of their native promoters, in the case of expression of eukaryotic proteins or proteins with rare codons (Terpe, 2006).

Another problem with production of a given recombinant protein is when this protein performs a function that interferes with the growth and homeostasis of the host cell, causing slower growth rate, lower cell densities and even death (Doherty et al., 1995). This situation can be minimized by using plasmids with lower copy number or systems where control of the level of induction is possible (Kay Terpe, 2006).

Frequently, overexpression of recombinant proteins in the cytoplasm can be associated with the disadvantage of inclusion bodies (IBs) formation. This happens because in high level of protein expression, several hydrophobic regions are available to interact with similar regions, leading to protein instability and aggregates formation. Accumulation of these aggregates results in inclusion bodies formation (Singh & Panda, 2005). Some strategies employed to overcome IBs formation are related with lowering culture temperature in order to reduce production rate, co-expression of chaperones, fusion of the desired protein to a solubility enhancer (fusion partners), changing culture conditions, for example pH, or even changing the bacterial strain to be used. Moreover, inclusion bodies can be solubilized and refolded to obtain, in the end, functional and active proteins (Markides, 1996; Singh & Panda, 2005).

1.1.1.2. Secretion of recombinant proteins

Furthermore, proteins might also be exported from the bacterial cytoplasm to the periplasm, as means for facilitating correct folding (especially when disulfide-bond formation is necessary), for downstream process simplification, for correct processing of the N-terminal (Georgiou & Segatori, 2005), and for enhanced biological activity (Kleiner-Grote et al., 2018). The secretion of recombinant proteins to the periplasm can only be possible if a signal peptide is fused in the N terminal, that allows recognition by components of the secretion pathways. Afterwards, once the protein reaches the periplasm, cell lysis is no longer necessary hence the protein can easily be secreted to the extracellular medium by osmotic shock or cell wall permeabilization, without being contaminated with cytoplasmic host proteins (Shokri et al., 2003). However, there are still some problems associated with heterologous protein production in the periplasmic space, which are incomplete translocation across the inner membrane, degradation of proteins by proteases and the inability of the export machinery to attend the high demand for exportation.

When the capacity is surpassed, the excess of expressed recombinant protein that didn't go through the inner membrane might accumulate in inclusion bodies. Only by carefully balancing the promoter strength and the gene copy number, will the expression level be optimized (Mergulhão et al., 2005; Terpe, 2006).

As mentioned above, contamination of the desired recombinant protein can be minimized if extracellular secretion is promoted, since *E. coli* does not normally excrete proteins to the culture medium, and extracellular proteolytic activity is considerably reduced. There are different ways by which proteins can reach the extracellular space; whether by the typically used mechanisms of *E. coli* (type I and type II secretion mechanisms), which are normally involved in pathogenicity for gram-negative bacteria, or by periplasmic leakage. Type I secretion mechanism is characterized by the transport of proteins directly from the cytoplasm into the medium. This mechanism is typically used by the cell to secrete toxins, proteases and adhesins into the extracellular space (Costa et al., 2015; Kleiner-Grote et al., 2018). In type II mechanism proteins are transported in a two-step fashion, they are firstly translocated across the inner membrane and then transported through the outer membrane. Translocation to the periplasm can occur via the Sec- (general secretory) or Tat- (twin-arginine translocation) pathways, depending on the use of specific signal sequences. Using the Sec pathway, proteins can either be targeted to the Sec translocase after translation (SecA/SecB-dependent) or alongside translation (SRP pathway). The tat pathway can transport folded proteins across the inner membrane (Costa et al., 2015; Kleiner-Grote et al., 2018; Mergulhão et al., 2005).

Periplasmic leakage might be of some importance for extracellular secretion. Recombinant protein production can induce alterations to the membrane structure, increasing its selective permeability and facilitating leakage. Furthermore, the choice of an appropriate strain may also be responsible for the success of the secretion. Leaky strains, which are mutant strains with interferences in the synthesis of outer membrane components, have been explored for this field. The problem is that the use of leaky strains may not be suitable for industrial production since they are reportedly growth impaired and lack the necessary robustness for high-density fermentations (Kleiner-Grote et al., 2018; Mergulhão et al., 2005; Yang et al., 2018).

In order to overcome some of these obstacles several research groups developed optimization strategies that allowed them to obtain higher quantities of the target protein. For instance, Zou et al. 2014 developed process optimization strategies to improve extracellular production of recombinant pullulanase from *Bacillus deramificans* in *E. coli*. They implemented a fed-batch fermentation strategy for high-cell-density cultivation, with gradual lactose addition for protein expression induction and determined the optimal lactose feeding rate and induction point. In addition, a glycine feeding strategy was formulated to promote the secretion of the recombinant protein. In the end, they obtained higher protein activities than the observed under unoptimized conditions. Wang et al. 2014 developed a process optimization of high-level extracellular production of alkaline pectate lyase in *E. coli* BL21 (DE3). They included in their work a multi-step glycerol feeding strategy to achieve high cell density cultivation, evaluated the extracellular secretion performance by inducing the bioreaction process at different temperatures and implemented a lactose continuous feeding strategy. All these strategies contributed to obtain high-level extracellular protein production.

1.2. Expression vectors

The expression vectors most commonly used nowadays are the result of several combinations of replicons, promoters, selection markers, multiple cloning sites, and fusion protein/fusion protein removal strategies, as it is depicted in figure 1.

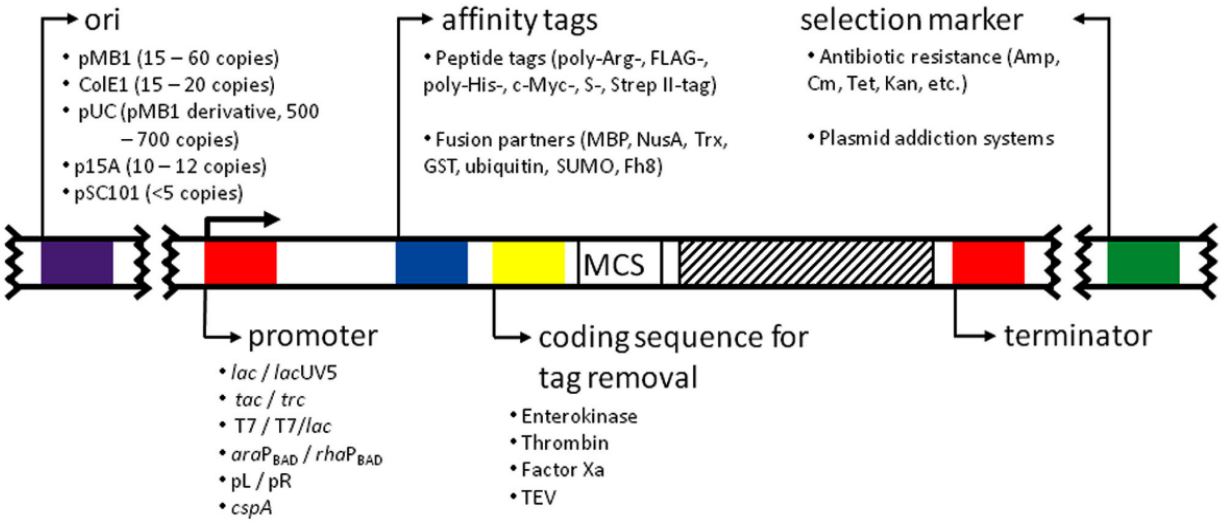


Figure 1 – Anatomy of an expression vector. The figure shows the major features present in common expression vectors (Adapted from Rosano and Ceccarelli 2014).

Genetic elements that go through replication need to contain a replicon, which consists of one origin of replication with its associated *cis*-acting control elements. This feature is responsible for the control of copy number of a given plasmid. A high plasmid number may impose a metabolic burden that reduces the bacterial growth rate and produce plasmid instability (Bentley et al., 1989; Birnbaum & Bailey, 1991). Consequently, using high copy number plasmids does not necessarily result in an increase in production yields. Additionally, use of low copy number plasmids can be an advantage if the presence of a high dose of a cloned gene or its product produces a deleterious effect to the cell. In the case that two recombinant proteins are being expressed using two plasmids, the chosen plasmids have to belong to different incompatibility groups, so that they can both be propagated in the cell, and no competition for the replication machinery is needed (Camps, 2010).

The *lac* promoter is probably the promoter with the most accumulated knowledge for its extended use in expression vectors. Belonging to the *lac* operon, when lactose is present, the induction of the system is promoted and the sugar can be used for protein production. Because this promoter does not function in the presence of glucose, a mutant with the *lac*UV5 promoter was developed (Lanzer & Bujard, 1988). However, since a considerate disadvantage associated with these promoters is the high level of expression in the absence of inducer. Therefore, promoter systems like the *tac* were developed and are reportedly quite stronger than the *lac*UV5 promoter (de Boer et al., 1983). The T7 promoter system is

present in the expression vectors series pET and the *ara*P_{BAD} in the pBAD vectors, whose characteristics are further explained below.

In order to separate the cells with the desired plasmid from the cells that do not contain the plasmid, a resistance marker is added to the plasmid backbone. Normally, in *E. coli* antibiotic resistance genes are used with this purpose, with the most common feature being resistance to ampicillin (Rosano & Ceccarelli, 2014). The plasmid contains the *bla* gene that encodes a periplasmic enzyme, the β-lactamase, which is responsible for inactivation of the β-lactam ring of β-lactam antibiotics, such as ampicillin. This mechanism allows the cells that contain the gene to propagate, whereas plasmid-free cells will decrease in number. The disadvantage with this type of systems is that as β-lactamase is continuously secreted, degradation of the antibiotic is carried out until its complete depletion from the medium (Korpimäki et al., 2003). Consequently, cells that do not carry the resistance gene will be able to grow. Tetracycline, on the other hand, has been shown to be stable during cultivation, as resistance relies on active efflux of the antibiotic from resistant cells (Roberts, 1996). Nowadays, some of the major concerns around large-scale cultures are the cost of antibiotics and the dissemination of antibiotic resistance across microbes. To overcome these problems, researchers have been focusing on the development of antibiotics-free plasmid systems. Based on the concept of plasmid addiction, an essential gene to the cells is deleted from the bacterial genome and placed in a plasmid. Consequently, after cell division, bacteria that do not contain the plasmid will not be able to survive (Peubez et al., 2010).

With the objective of obtaining a highly purified active recombinant protein, the expression of a peptide tag (few amino acids) or a fusion partner (large polypeptide) simultaneously with the desired protein is essential for protein detection throughout the process, for maximum solubility and/or for an easier purification process. The advantage associated with the use of small peptides is explained by lower interference when fused to the protein. However, in some cases, they can have negative effects on the tertiary structure or biological activity of the chimeric protein (Bucher et al., 2002; Khan et al., 2012; Klose et al., 2004). Some examples of small peptide tags are poly-His-, poly-Arg-, S-, and Strep II-tags (Terpe, 2003). Because antibodies against these tags are commercially available, the produced proteins can easily be detected by Western Blot. Additionally, these tags can also help in purification processes, as the recombinant proteins can bind tightly and specifically to the designed ligands present in resins, through these tags. For example, His-tagged proteins can be recovered by metal ion affinity chromatography using columns loaded with either Ni^{2+} or Co^{2+} nitriloacetic acid-agarose resins (Riguero et al., 2020). Alternatively, addition of a non-peptide fusion partner can work as an enhancer of protein solubility (Hammarström et al., 2009). Some of the most used fusion partners are maltose-binding protein (MBP) (Kapust & Waugh, 1999), N-utilization substance protein A (NusA) (Davis et al., 1999), thioredoxin (Trx) (LaVallie et al. 1993) and glutathione *S*-transferase (GST) (Smith & Johnson, 1988).

When the produced recombinant protein needs to go through structural or biochemical studies, the fusion partners and peptide tags need to be removed as they can interfere with protein activity and structure. One available strategy is the enzymatic cleavage of the tag that requires the presence of a sequence that encodes for protease cleavage sites in the expression vector. Enterokinase, thrombin,

factor Xa and tobacco etch virus (TEV) protease are examples of enzymes successful for removal of peptide tags and fusion partners (Blommel & Fox, 2007; Jenny et al., 2003). TEV has become the most used protease against His-tags, because of its high specificity and ease of production in large quantities and also because it only leaves a serine or a glycine residue after digestion. Chemical cleavage involves treatment of the tag with a chemical reagent, for example cyanogen bromide (CnBr). Although these reagents are cheap in comparison with proteolytic enzymes and their elimination from the reaction mixture is easy, the reaction conditions are harsh and might often cause unwanted protein modifications (Hwang et al., 2014).

1.2.1. pET-23a(+), pET-22b(+) and pMLBAD expression vectors

Expression vectors belonging to the pET series are very commonly used for the production of recombinant proteins in *E. coli*. Figure 2 (left) depicts the backbone of the pET-23a(+) expression vector (Novagen), which possesses the pMB1 origin, allowing for 15-60 copies of the vector in each cell. An ampicillin resistance gene is present in the vector, functioning as the selectable marker. Expression is controlled by a T7 promotor system, where the gene of interest is cloned downstream of a promoter recognized by the phage T7 RNA polymerase (T7 RNAP). This highly active polymerase needs to be provided in another plasmid or is already present in the genome of the bacterial strain in use. As *E. coli* BL21 (DE3) already contains this feature in its genome, under the transcriptional control of a *lac*UV5 promoter, the system can be induced and expression is started by the addition of lactose or its non-hydrolysable analog isopropyl β-D-1-thiogalactopyranoside (IPTG), (Rosano & Ceccarelli, 2014). Another example of an expression vector from the pET series is the pET-22b(+), also depicted in figure 2 (right). The principal characteristics of this expression vector are very similar of those of the pET-23a(+), with the main difference being the presence of a N-terminal signal sequence *pelB* for potential periplasmic localization (Novagen).

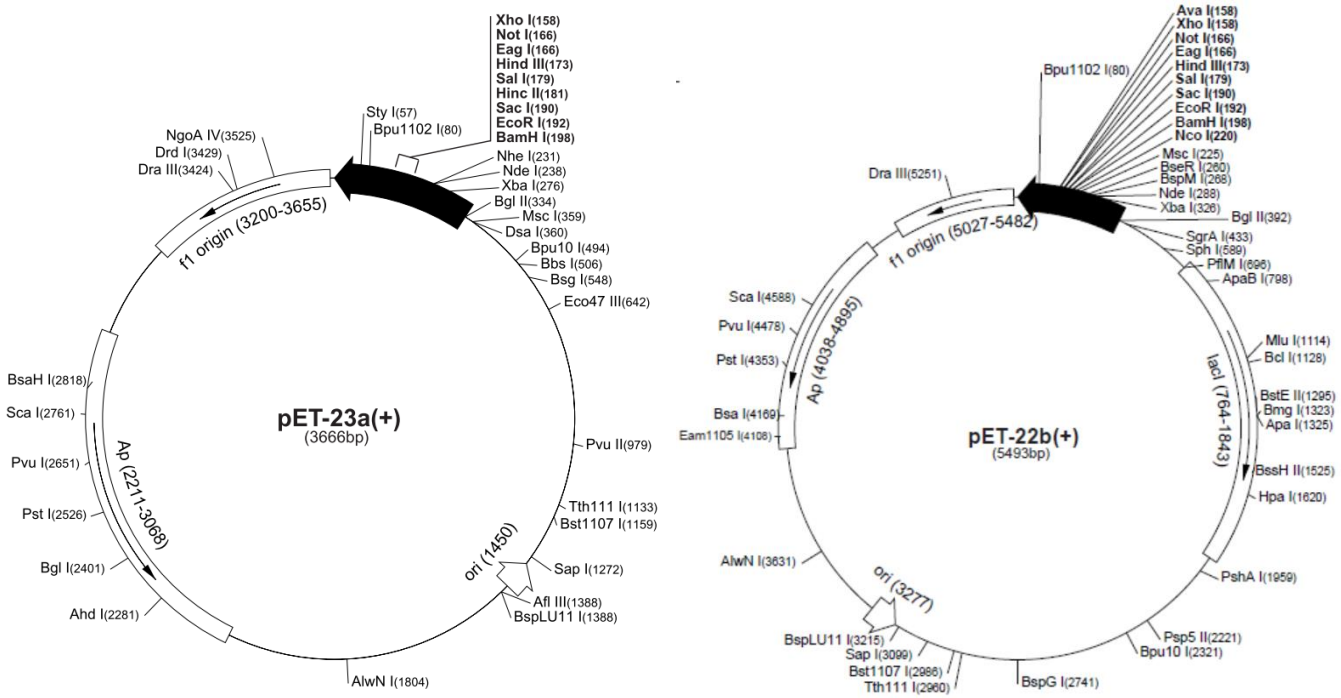


Figure 2 – Backbone of the pET-23a(+) (left) and pET-22b(+) (right) expression vectors from Novagen (Adapted from Novagen).

The pMLBAD expression vector was originally developed by Lefebre and Valvano 2002, for gene expression in *Burkholderia cepacia* isolates. This vector resulted from the combination of a pBBR1 plasmid derivative, the pME6000, carrying several important features, shown in figure 3. The mobilization (*mob*) region, a feature from the original plasmid, encodes a putative protein that is necessary to mediate transfer of the plasmid when transfer functions are supplied in *trans*. Additionally, the plasmid also contains a *rep* region, that encodes a replication protein essential for a stable plasmid maintenance. Regarding the promoter sequences, as it is possible to see below, an arabinose inducible BAD promoter sequence (pBAD) was placed upstream of a multiple cloning site (MCS). This promoter system, together with a AraC protein functioning as repressor and activator, allows that in the absence of L-arabinose, AraC is repressing transcription by binding to two different sites in bacterial DNA, hence forming a loop that prevents binding of the RNA polymerase to the promoter. When arabinose is added, AraC becomes active and promotes transcription of the *ara* promoter. The *dhfr* gene encodes for resistance of the plasmid to trimethoprim antibiotic, allowing selection of this plasmid containing-cells (Lefebre & Valvano, 2002; Rosano & Ceccarelli, 2014; Terpe, 2006).

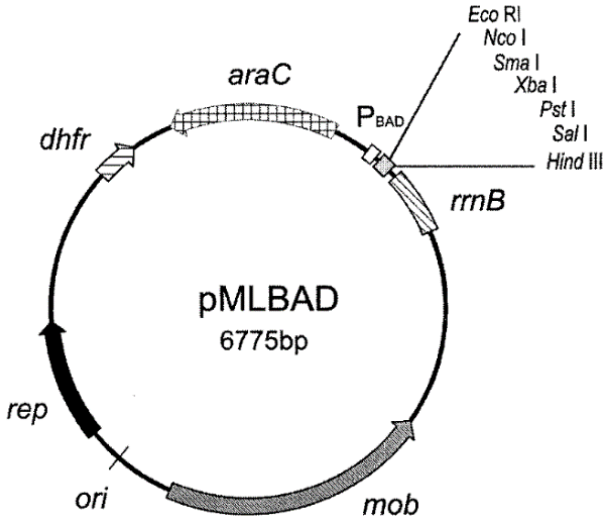


Figure 3 – Backbone of the pMLBAD expression vector (adapted from Lefebre and Valvano 2002).

1.3. Braun's Lipoprotein (Lpp)

Gram-negative bacteria are characterized by having a complex macromolecular structure serving as an envelope to the cell, where three different layers can be observed: the cytoplasmic membrane, or inner membrane (IM), the outer membrane (OM), serving as interface to the environment and the peptidoglycan (PG) sacculus (Asmar & Collet, 2018). This last layer has a form similar to a net, and is located between the cytoplasmic membrane and the outer membrane. The PG is a polymer composed of glycan chains (*N*-acetylglucosamine [NAcGlc]-*N*-acetylmuramic acid [NAcMur] disaccharides), and cross-linked peptides (pentapeptides bound to the NAcMur units). The outer membrane relies on top of this solid layer and is promptly linked through Braun's lipoprotein (Lpp, the only covalent bond to the PG Network (PGN)) and other lipoproteins (for example, the PGN-associated lipoprotein [Pal]). All these characteristics provide shape and structure to the cell, the capacity to counteract osmotic pressure and also offer some resistance to the diffusion of substances (Juan et al., 2018). Thus, bacterial virulence and viability depend on the stable synthesis, maintenance and structure of the peptidoglycan, since it has been shown before that defects in the structural lipoproteins can result in the loss of integrity of the outer membrane, allowing periplasmic protein leakage and formation of vesicles (Cascales et al., 2002; Juan et al., 2018; Sha et al., 2008). Braun's lipoprotein was originally identified in *E. coli* (Sha et al., 2008), is the most numerous protein in this bacteria, with about 700 000 copies per cell, and contributes significantly to the rigidity of the cell envelopes (Ni et al., 2007). Because Lpp is so abundant, cells need to turn down its synthesis if the assembly of the bacterial cell envelope encounters some problems. This control is exerted at the post-transcriptional level, since blocking translation seems to be a more efficient mechanism to rapidly lower the amount of Lpp molecules than transcriptional repression, given the high stability of the Lpp mRNA (Asmar & Collet, 2018). Structurally, the Lpp Braun Lipoprotein contains 58 residues in its primary sequence, with a molecular weight of 5.8 kDa. Folds into an α-helical trimeric coiled-coil structure and is characterized as having three domains: an N-terminal capping motif, a long

(residues 5-53) coiled-coil domain formed by three parallel α helices and a hydrophobic helix-termination motif (Asmar & Collet, 2018).

Lpp does not only play a structural role in the cell envelope, but is also quite important for bacterial pathogenesis, either by being directly or indirectly responsible for virulence of the pathogen or by modulating the immune response of the host (Asmar & Collet, 2018). For instance, in the pathogenic *E. coli* strain O157:H7, the deletion of the *lpp* gene resulted in the reduction of cell invasiveness by attenuating biofilm formation (Uhlich et al., 2009). Furthermore, defects in *Yersinia* Braun's lipoprotein have been related also to an attenuation of motility and invasiveness, suggesting that the knockout (KO) mutants might be useful tools in the development of potential vaccine candidates for bubonic and pneumonic plague (Juan et al., 2018; Sha et al., 2008).

As it is possible to infer, modifications of the structural proteins of the peptidoglycan have consequences probably related with loss of outer membrane integrity, resulting in permeability barrier loss and deficient performance of the anchored lipopolysaccharide, leading to the decrease in the capacity to resist host's immune response and loss of adherence and motility (Juan et al., 2018). Additionally, *E. coli* cells deleted for *lpp* also exhibited an increased outer membrane permeability, making them able to export recombinant proteins directly to the extracellular medium, facilitating protein detection and purification (Asmar & Collet, 2018; Shin & Chen, 2008). Ni et al. 2007 demonstrated that the enhanced permeability through the outer membrane is beneficial to whole-cell bioprocess in which product permeability was a challenge, since the expressed proteins could be recovered directly from the growth medium without cell lysis. The deletion did not affect the cell growth and metabolism in a significant way nor affected the cell's ability to express recombinant proteins. Therefore, this contributes to the idea that the deletion mutant could possibly be suitable for several biotechnological applications (Ni et al., 2007).

1.4. Antisense RNAs

The use of short fragments of nucleic acids, denominated antisense oligonucleotides (ASO) as therapeutic agents or to alter gene function, has achieved a certain relevance due to the simplicity of the systems, inexpensive cost and rational design (Dias & Stein, 2002). The principle behind this technology lies on the fact that a sequence, with complementarity to a certain mRNA, is capable of modulating the levels of such RNA and consequently interfere on the translational levels of the encoded protein (Bennett, 2019; Dias & Stein, 2002). If the protein being targeted is essential for cell growth and maintenance, then the antisense agent would be capable of slowing down these processes (Stein & Cheng, 1993). This is normally very useful when the target proteins are very difficult to affect using conventional small-molecules or protein-based strategies (for example, adapter proteins, transcription factors, etc.) (Bennett, 2019).

In order for the antisense oligonucleotide and other antisense compounds to anneal to an mRNA or a functional RNA, such as ribosomal RNA, the RNA target region needs to be accessible. When the

annealing takes place, various mechanisms of disruption can occur. Disruption of mRNA translation as a result of steric hindrance can happen only if the target molecule is a mRNA, either by blocking the access of the ribosome to the mRNA or blocking ribosomal readthrough. Other mechanisms, such as, fast degradation of duplex RNA and hybrid RNA/DNA duplex or cleavage of the target RNA by the antisense compound itself can occur if the target molecules are mRNAs or functional RNAs (Rasmussen et al., 2007).

Numerous factors can affect the inhibitory efficiency of the hybridization strategies mentioned. These include the length and structure, the binding rate, the intracellular concentration and the resistance to degradation of the chosen antisense molecule. For instance, antisense molecules need to be designed with the objective of targeting the start codon and the Shine-Dalgarno sequence of a mRNA, since this is the region available for ribosome assembly. This region is the usual target for antisense inhibition experiments, and normally results in success (Rasmussen et al., 2007).

The most basic form of an antisense RNA (asRNA) is an unmodified RNA molecule of the exact reverse and complement sequence of the target RNA, as it is possible to observe in figure 4. To construct the antisense, a segment of the target gene is amplified and cloned into a vector in an antisense orientation, downstream of an inducible promoter. Following transcription of the asRNA-encoding vector, the antisense molecule is now available to act on the target molecule, either by sterically blocking translation or by the rapid degradation of the target RNA, by RNases specific for double-stranded RNA (dsRNA). Several studies have shown that the longer the antisense and the longer the target hybridization, the inhibition will also be greater, verifying the existence of a positive correlation between these features (Rasmussen et al., 2007). The asRNA has mainly been used to study gene function. For instance, when the gene target encodes an essential protein and the knockout mutants will not be able to proliferate, or when an asRNA encoded by a plasmid provokes the downregulation of a specific gene, functional studies can be conducted (Chen et al., 2003). AsRNAs can also be used as metabolic engineering tools, in order to improve the bacterial cells capacity as fermenters and hosts for recombinant heterologous protein production (Rasmussen et al., 2007).

In addition to the antisense RNA previously mentioned, figure 4 also demonstrates how antisense oligonucleotides can be used to target mRNA (Rasmussen et al., 2007). These include antisense phosphodiester oligodeoxyribonucleotides (ODN), phosphorothioate ODNs (PS-ODN) usually short sequences of about 10-30 nucleotides of synthetic DNA, complementary to a given mRNA target. Once hybridization occurs, the duplex is recognized by RNase H and the RNA strand is degraded. On the other hand, Peptide Nucleic Acids (PNAs) and Phosphorodiamidate Morpholino Oligomers (PMOs), act by steric-blocking of ribosomal assembly and hence translation (Hegarty & Stewart, 2018; Rasmussen et al., 2007).

Also depicted in figure 4 are the mechanisms of action of external guide sequences (EGS) and catalytic antisense. The first ones are small asRNA molecules that hybridize to their target RNA to create a structure similar to the naturally occurring substrate of RNase P, responsible for the cleavage of the target molecule. The catalytic antisense sequence contains a self-cleaving domain responsible for the cleavage of the target RNA after hybridization (Rasmussen et al., 2007).

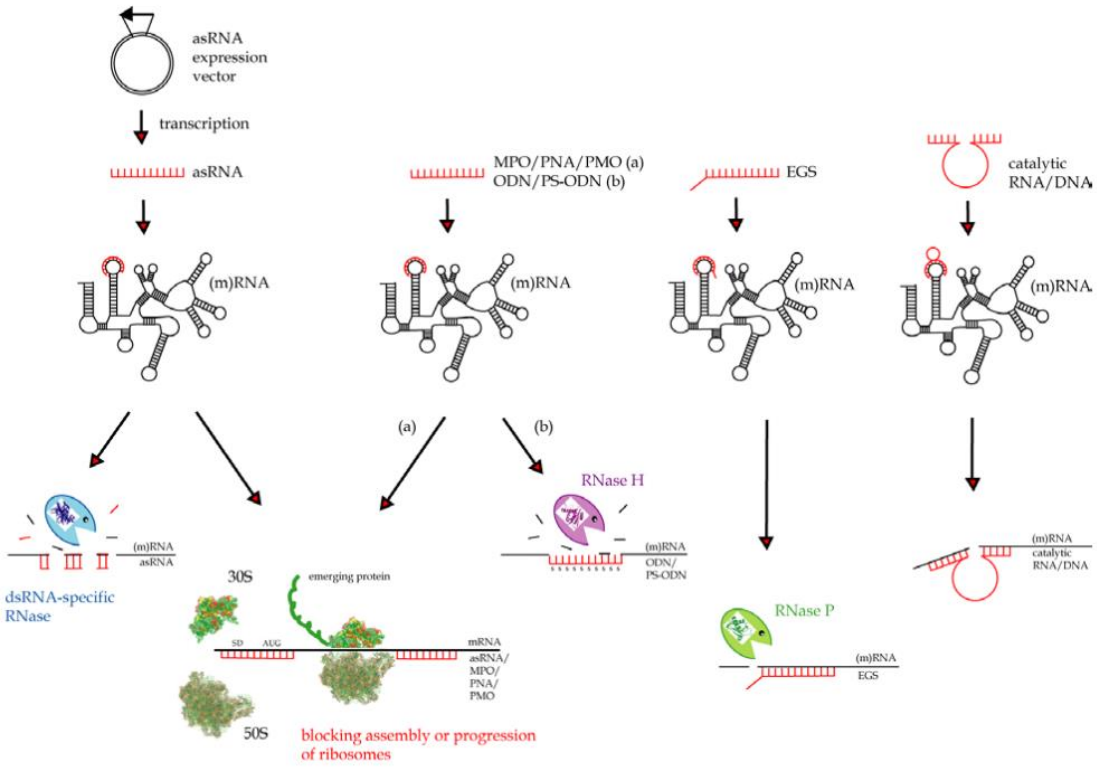


Figure 4 – Mechanisms of action for antisense inhibition. Inhibition mechanism by AsRNA includes steric hindrance of mRNA translation or rapid degradation of target RNA possibly by dsRNA-specific RNases. MPOs, PNAs and PMOs also inhibit expression by sterically blocking translation. ODNs and PS-ODNs induce cleavage of target RNA by RNase H, while EGSs guide RNase P in target RNA cleavage. Catalytic RNA or DNA cleaves the target RNA upon hybridization (adapted from Rasmussen et al. 2007). Abbreviations are explicit in the text.

1.5. Aquaculture

Aquaculture involves the culture of all forms of aquatic animals and plants in fresh, brackish and marine environments (Defoirdt et al., 2007). It is the sector of production of food by animals with more rapid growth than all other food sectors. From 1970 to 2001, the contribution of aquaculture for global supplies of fish, crustaceans and molluscs, increased from 3.9% to 29% of total production, respectively (Sommerset et al., 2005). Whereas, during the period 2000 to 2016, this sector observed an average annual growth rate of 5.8% (Ahmed & Thompson, 2019).

This industry has become an important source of seafood since the capture fishing industry has declined and wild stocks are disappearing. The tendencies are, that all fish farming will become industrialized due to the increasing investment in this industry (Sommerset et al., 2005). Sustainable

aquaculture is limited by a range of concerns, resulting from impacts of this sector inputs and resources on the environment, including land, water and energy. In order to develop a more long-term sustainable production, a proper management of resources is needed (Ahmed & Thompson, 2019). However, as it was considered by the United Nations' Food and Agriculture Organization (FAO), disease outbreaks (Defoirdt et al., 2007), habitat destruction and water pollution, among others, pose a considerable disadvantage to the development of the aquaculture sector, reflecting on monetary losses (Ahmed & Thompson, 2019).

1.5.1. Vibriosis

Certain strains of *vibrios* are harmful to aquaculture settings due to their pathogenic effects, where production of fish must be high. To maintain productivity, high inputs of feeding products, water changes and massive use of antibiotics need to be implemented and these conditions seem to favor proliferation of *vibrios* and contribute to their virulence and disease prevalence. Consequently, the presence of these pathogens in aquaculture has catastrophic effects to the environment (Thompson et al., 2004).

Vibrio spp. are gram-negative bacteria, usually have the shape of motile rods, are mesophilic and chemoorganotrophic, and have a facultatively fermentative metabolism. They belong to the *Gammaproteobacteria* class and to the *Vibrionaceae* family, which comprises many genera including *Vibrio*. These bacteria are found in aquatic, estuarine and freshwater environments, in sediments and forming either mutualistic or pathogenic associations with plankton and marine organisms (Huehn et al., 2014) and are characterized by being metabolically versatile (Le Roux & Blokesch, 2018). In addition, *Vibrio* spp. usually are able to communicate between cells, by a mechanism known as quorum sensing, and that allows many interactions with a variety of organisms (Le Roux & Blokesch, 2018). For example, *Vibrio harveyi*, *Vibrio splendidus* and *Vibrio tubiashii*, have been associated with disease in different species of fish and shellfish, while the *Vibrio halioticoli* group is formed by species with mutualistic relationships to abalones (Thompson et al., 2005).

Disease caused by *Vibrio* is mostly described as vibriosis and result in lethargy, tissue necrosis or slow growth (Defoirdt et al., 2007). In many cases, *Vibrio* spp. are opportunistic pathogens, meaning they cause disease in host organisms by overwhelming the host's defense mechanism that are already immune suppressed or physiological stressed (Munn, 2015), with the infections being associated with intensive culture and adverse environmental conditions of growth. Reports show that, for example, in Indonesia, losses due to disease caused by these species in culture are considerable. In addition, certain species can also cause human infections, for example, *Vibrio cholerae*, which is the causative agent of cholera (Defoirdt et al., 2007).

To surpass the problems that these species were causing, industry started to take advantage of antibiotics. However, the uncontrollable use of antibiotics led to the increase of *Vibrio* strains with multiple resistance to several antibiotics, making them ineffective against Vibriosis (Defoirdt et al., 2007). Apart from that, the excessive use of antibiotics in aquaculture also poses a dangerous threat to human

health and to the environment. The antibiotic resistance determinants have been shown to be transmitted by horizontal gene transfer to several animal and human pathogens. If residual levels of antibiotics are present in aquaculture products in the market, this can lead to an alteration of the normal human gut microflora, resulting in problems of allergy and toxicity (Defoirdt et al., 2007). For instance, Albuquerque Costa et al. 2015 conducted a study where they concluded that cultured shrimps are possible vehicles of vibrios resistant to β-lactam and tetracycline. Thus, new strategies to control pathogenic bacteria impact in aquaculture and make the industry more sustainable are urgent (Defoirdt et al., 2007).

One important strategy that is currently being developed is the stimulation of host defenses, this way increasing resistance to infectious disease. For instance, vaccination is capable of promoting specific immune responses in the host, with the aim of conferring long-lasting protection through immunological memory, and immunostimulation which enhances nonspecific defense systems (Defoirdt et al., 2007; Sommerset et al., 2005).

1.5.2. Protein-based vaccines against Vibriosis

Vaccines may mediate protection through multiple mechanisms involving the induction of specific antibodies. The primary goal of vaccination is to induce humoral memory through creation of antigen-specific plasma cells and antigen-experienced memory B cells (Xu & Kulp, 2019). Most vaccines commercially available rely on either inactivated (killed) or live attenuated (weakened) technologies, which have been used to address many of the important veterinary and human diseases with success. However, they have several limitations and potential problems associated with the development of protective immunity without causing severe disease symptoms in the host. Many researchers have set out their goal to produce large quantities of a highly purified immunogenic protein to generate safe and effective vaccines, once the protein's sequence is identified. With the emergence of recombinant DNA technology, foreign genes could be inserted into expression vectors, introduced into cells acting as "production factories", allowing the production of the encoded proteins to take place. This approach represents a relatively inexhaustible and cheap source of protein from the infectious agent to be applied in vaccination studies. *E. coli* bacteria were the first recombinant expression systems to be established for this purpose due to the relatively large quantities of proteins and ease of production they can offer (Francis, 2018).

Bacteria from the *Vibrio* species contain many different outer membrane proteins (OMPs), which possess a highly immunogenic capacity due to the presence of exposed epitopes on the cell surface (Y. D. Li et al., 2010). Inoue et al. 1995a discovered OmpK as a 26-kDa OMP, commonly present in many *Vibrio* and *Photobacterium* species, that was firstly discovered due to the isolation of a virulent vibriophage, the KVP40, in *Vibrio parahaemolyticus* 1010. This vibriophage exhibited a broad host specificity present in at least eight *Vibrio* species, which indicated the presence of a common cell surface component serving as the receptor for this phage – the OmpK (Inoue et al., 1995a). After cloning and sequencing of the gene encoding the OmpK protein, results indicated that it consists of 789 nucleotides

encoding 263 amino acids. Since the first 20 amino acids might correspond to the signal peptide, the mature protein would contain 243 amino acids and a calculated molecular mass of 27 458 Da (Inoue et al., 1995b).

Previous investigation has concluded that some *Vibrio* species OMPs, such as OmpW, OmpV, OmpK and OmpU, have the ability to induce protective immunity (Hong et al., 2006). Analysis of the amino acid sequence of these proteins has revealed high identity among many different *Vibrio* species, indicating that these proteins possess similar biological function (Y. D. Li et al., 2010). In addition, it was discovered that OmpK protein is likely to be a genus-specific antigen of the *Vibrio* species, making this finding helpful in facilitating future rapid detection of presence of these pathogens and the development of protective vaccines against *Vibrio* (Y. D. Li et al., 2010).

The protective capacity of OmpK has been reported in *V. harveyi* when orange-spotted groupers (*Epinephelus coioides*) were vaccinated with the recombinant OmpK, after infection with the pathogen (Ningqiu et al., 2008). In addition, large yellow croakers (*Pseudosciaena crocea*) that were vaccinated with OmpK from *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*, showed a high-level protection against the infection by corresponding pathogens (Mao et al. 2007; Qian et al., 2008; Zhang et al., 2007). Polyclonal antibodies raised against the recombinant OmpK from *V. harveyi* recognized OmpK homologues from other strains of *Vibrio* species by immunoblotting, contributing to the fact that this antigen sequence is highly conserved and shared among different species (N. Li et al., 2010).

Silvaraj et al. 2020 conducted a study in which they verified that immunization of fish with a vaccine containing the recombinant OmpK protein induced immune responses in an effective way that eventually protected the fish against pathogen challenge. Analysis to the serum and gut of vaccinated fish resulted in the presence of high quantities of antibodies as early as day 7 post-vaccination. These results suggested that recombinant OmpK is a valid candidate vaccine molecule against several *Vibrio* species.

Subunit vaccines refer to the extraction or production of specific protein structures of bacteria, most likely vaccines made of epitopes (Liljeqvist & Ståhl, 1999). It is possible, by genetic engineering, to transfer the antigen-encoding genes to another microorganism different from the target pathogen, in order to produce the immunogenic proteins of *Vibrio* species (Hamod et al., 2012). This type of vaccines often present strong stability, stimulate the production of a specific immune response and can be used for vaccine research in the case the pathogens are difficult to cultivate or have potential carcinogenicity. Ultimately, vaccination is a method superior in high efficiency, safety and convenience, contributing to obtain greater economic benefits and making it easier to control fish diseases (Ji et al., 2020).

2. Introduction to the thesis theme

A 2D image of the OmpK structure predicted using Pred-TMBB software by João Lopes in 2017 is represented in figure 5. Using bioinformatic tools, the discovery of some regions with high immunogenic capacity, represented by the amino acids on the extracellular space, was possible (Lopes, 2017). The production of the complete OmpK protein was also developed and many different purification strategies were tried in order to obtain a successful recovery of the protein. Furthermore, a plasmid containing the sequence of the lpp antisense with the objective of increasing the outer membrane permeability of the *E. coli* expressing cells, was developed and some physiological tests were carried out to see the effect of the plasmid on the cells. Additionally, a new strategy of producing the OmpK protein was carried out where only an antigenic fragment of the whole protein was produced and several different attempts of overexpression were tried (Reisenbauer, 2018).

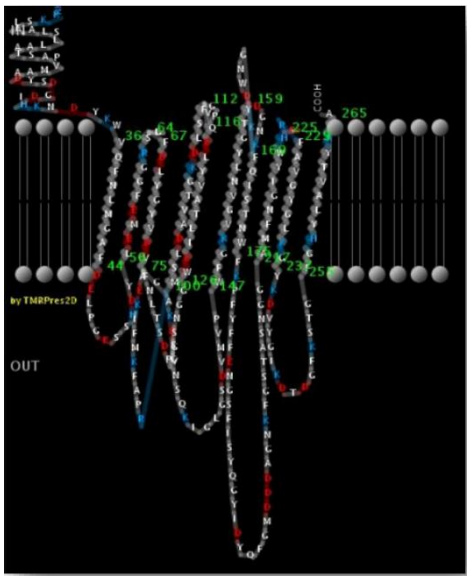


Figure 5 – 2D image of the protein structure of OmpK predicted by Pred-TMBB (Adapted from Lopes 2017).

This thesis work is a continuation of previous MSc thesis (Lopes, 2017; Reisenbauer, 2018), and was developed at iBB-IST. The primary objective was the development of an *E. coli* BL21 (DE3) strain with an improved capacity for secretion of recombinant heterologous proteins to the extracellular medium. For this, a lpp antisense oligonucleotide was constructed and the effect of this molecule on the cellular viability and productivity was be evaluated, by monitoring the cell growth of *E. coli* BL21 (DE3) through time and evaluating the effect the antisense can be causing by testing the susceptibility to stressors. An RT-PCR experiment was carried out to allow the evaluation of the levels of the lpp antisense in the cultured cells throughout cell growth. The levels of the inducer L-(+)-Arabinose were also evaluated by HPLC to follow its consumption by the cells, in different conditions. Furthermore, an OmpK fragment 21-99 derived from the OmpK of *Vibrio alginolyticus*, a fish pathogen that infects soles in Portuguese aquacultures, was constructed, cloned into the pET-22b(+) plasmid and different peptide overexpression strategies were carried out in order to understand what conditions would be best to

produce elevated quantities of the OmpK fragment. Consequently, the bacterial strain produced was used as a host for the production of the recombinant peptide.

3. Materials and Methods

3.1. Bacterial strains, plasmids and culture conditions

Table 1 contains the bacterial strains and plasmids used in the present work.

Table 1 – List of the bacterial strain and plasmids used during the experimental work, as well as the description and the source of the materials.

Strain/Plasmids	*Description or genotype*	Source
Strain		
***Escherichia coli* BL21 (DE3)**	B F^- *ompT hsdS* (r_B^- m_B^-) *dcm gal* λ(DE3)	Stratagene
***E. coli* αDH5**	F^-, φ80 *lac*ZΔM15 *end*A1 *rec*A1 *hsd*R17(r_K^- m_K^+) *sup*E44 *thi*-1 Δ*gyr*A96 (Δ*lac*ZYA-*arg*F) U169 *rel*A1	Invitrogen
Plasmids		
pMLBAD	pBBR1 *ori*, *araC*/P_{BAD}, *mob*$^+$, Tp^r	Lefebre and Valvano (2002)
pET23a+	pBR322 origin, T7 promoter, C-terminal 6x His-Tag, Ap^r	Novagen
pET-22b(+)	pBR322 origin, T7 promoter, pelB coding sequence, C-terminal 6x His-Tag, Ap^r	Novagen
pSAS39	pMLBAD containing *E. coli* BL21 lpp antisense sequence	This thesis
pJBF2	pET23a+ containing the OmpK gene from *V. alginolyticus*	Lopes (2017)
pFCN1	pET-22b(+) containing OmpK encoded aminoacid fragment 21-99 from *V. alginolyticus*	This thesis

While being used, *E. coli* strains were maintained in Lennox broth (LB) agar plates (composed of 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 20 g/L agar). The plates containing the cells transformed with the pSAS39 or pMLBAD plasmid, were supplemented with 100 µg/mL of trimethoprim (Tp) and when transformed with pET-22b(+), pJBF2 or pFCN1, were supplemented with 150 µg/mL of ampicillin (Ap). The liquid cultures were carried out in LB liquid medium (composed of 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, diluted in distilled water), properly supplemented, when necessary, at 37 ºC and 250 rpm of orbital agitation, unless otherwise mentioned.

3.2. Methods for insertion of foreign DNA in bacterial cells

3.2.1. Preparation of competent *E. coli* αDH5 cells and transformation by heat shock method

A colony of *E. coli* αDH5 cells was grown overnight in 6 mL of LB liquid medium at 37 ºC, at 250 rpm of orbital agitation. The overnight culture was used to inoculate 100 mL of LB liquid medium by transferring aliquots to an Optical Density at 640 nm (OD_{640}) of 0.1, followed by incubation at 37 ºC and 250 rpm. Once the bacterial culture reached an OD_{640} of around 0.6, the cells were harvested by centrifugation in sterile centrifuge bottles at 5600 rpm (12145 rotor, J2-21, Beckman), for 5 minutes at 4 ºC. Then, the cell pellets went through two washing steps in order to eliminate the salts. Firstly, 100 mL of $MgCl_2$ 0.1 M was used to resuspend the pellet, followed by centrifugation at 3400 rpm for 5 minutes at 4 ºC. Secondly, the cells were washed by adding 100 mL of $CaCl_2$ 0.1 M, resuspended and placed on ice for 30 minutes. The mixture was again centrifuged in the same conditions as before. Afterwards, the resulting cell pellet was resuspended in 22 mL of $CaCl_2$ 0.1 M and 3.5 mL of glycerol 86 % (w/v) was added. The resulting suspension was divided into 500 µL aliquots in 1.5 mL cryovials and kept at -80 ºC until further use.

To perform transformation, 150 µL of competent *E. coli* αDH5 cells were mixed with 50 µL of TCM buffer (10 mM Tris, 10 mM $CaCl_2$, 10 mM $MgCl_2$ pH 7.5) and 1 to 50 ng of the plasmid DNA construction or of the pET-22b(+), and placed on ice for 30 minutes. Afterwards, to achieve uptake of the exogenous plasmid DNA by the bacterial cells, they are permeabilized by heat shock at 42 ºC for 3 minutes, and then placed again on ice for 5 minutes. Next, for recovery of the bacterial cells from the previous treatment were added 800 µL of LB liquid medium and the suspensions were incubated at 37 ºC for 1 hour at 250 rpm of orbital agitation. The resultant cell suspensions were plated on LB agar plates, containing 150 µg/mL of Ampicillin (Ap150), and incubated at 37 ºC overnight. On the next day, several colonies from the plates were transferred to fresh plates with selective medium.

3.2.2. Preparation of electrocompetent *E. coli* BL21 (DE3) cells and transformation by electroporation

The preparation of electro-competent cells was initiated by inoculation of 30 mL of LB medium with a colony of *E. coli* BL21 (DE3), followed by incubation at 37 ºC and 250 rpm orbital agitation, around 16 hours. Then 100 mL of LB liquid medium was inoculated with an aliquot from the previous culture to an initial OD_{640nm} of 0.1 and incubated at 37 ºC and 250 rpm orbital agitation. When the bacterial culture reached the mid-log phase ($OD_{640nm} \approx 0.8$), the cells were harvested by centrifugation at 8000 rpm (VWR microstar 12), during 15 minutes at 4 ºC. After pellet resuspension with 100 mL of cold sterile distilled water, the cells were washed twice, with 60 mL and then 20 mL of cold distilled water and centrifuged in similar conditions between the washing steps. Then, the cellular sediment is resuspended in 4 mL of cold glycerol 10 % (v/v) and centrifuged at 8000 rpm (VWR microstar 12), during 15 minutes at 4 ºC. After discarding the supernatant, the pellet is resuspended in 2 mL of cold glycerol 10 % (v/v) and aliquots of 110 µL were placed in frozen eppendorfs and then stored at -80 ºC until further use.

Plasmid DNA solution (around 1 ng) was added to 110 µL of thawed electrocompetent *E. coli* BL21 (DE3) cells stock. The whole suspension was transferred to an ice-cold cuvette and then placed on the Gene Pulser apparatus (Biorad). The cuvette was then submitted to an electric pulse with a voltage of 2.5 kV, capacitance 25 µF and resistance 4 kΩ followed by addition of 800 µL of LB liquid medium and incubation for 1 hour at 37 ºC and 250 rpm. The cell suspension was then plated in selective medium agar plates (LB agar plates supplemented with 150 µg/mL of ampicillin) and incubated at 37 ºC overnight.

3.3. *E. coli* BL21 (DE3) lpp antisense

3.3.1. Construction of the plasmid pSAS39 for the induction of *E. coli* BL21 (DE3) lpp antisense

The procedure for the construction of the plasmid containing the inserted lpp antisense was conducted by the investigator Sílvia Sousa, following similar steps as previously described by Reisenbauer 2018 in her thesis work, since the construction of the plasmid needed to be repeated. Figure 6 shows the *E. coli* BL21 (DE3) lpp antisense sequence.

CTTGCGGTATTTAGTAGCCATGTTGTCCAGACGCTGGTTAGCACGAGCTGCGTC
ATCTTTAGCAGCCTGAACGTCGGAACGCATTGCGTTCACGTCGTTGCTCAGCTG
GTCAACTTTAGCGTTCAGAGTCTGAACGTCAGAAGACAGCTGATCGATTTTAGC
GTTGCTGGAGCAACCTGCCAGCAGAGTAGAACCCAGGATTACCGCGCCCAGTA
CCAGTTTAGTAGCTTTCAT

Figure 6 – *E. coli* BL21 (DE3) lpp antisense sequence.

Briefly, the oligonucleotide primers were designed based on the sequence in figure 6 and were synthesized by STAB VIDA (Portugal). A PCR experiment was performed to amplify the lpp antisense sequence in a 2720 thermal cycler (Applied Biosystems), using the designed primers Up_EcoRI_asLPP (5'- AAG AAT TCC TTG CGG TAT TTA G -3') and Lw_HindIII_asLpp (5'- AAA AGC TTA TGA AAG CTA CTA AAC -3'). The reaction mix for the amplification contained 5 ng DNA template, 0.02 U/µL Phusion® High-Fidelity DNA polymerase enzyme (Thermofisher), 1x of Phusion HF buffer (Thermofisher), 200 µM of each Deoxynucleotide Triphosphates (dNTPs) and 1.0 µM of each primer, for a total reaction volume of 20 µL. The samples were subjected to an initial denaturation at 98 ºC for 3 minutes, followed by 30 cycles of denaturation (98 ºC for 10 seconds), annealing (61 ºC for 30 seconds) and elongation (72 ºC for 8 seconds) and a final elongation step for 7 minutes at 72 ºC. After visualizing the PCR product on the agarose gel to check the purity and to see if it has the expected size, the band was extracted using the NZY Gelpure kit (NzyTech), according to the manufacturer's instructions and the amount of DNA purified was quantified. The extracted PCR product of the lpp antisense sequence and the pMLBAD plasmid were digested overnight with the restriction enzymes EcoRI and HindIII (Thermo Scientific) according to manual instructions.

The restricted samples went through ethanol precipitation of the DNA, to purify and concentrate the DNA samples, similar to what is explained in section 3.4.1.3. The EcoRI/HindIII restricted pMLBAD plasmid was mixed with the EcoRI/HindIII restricted lpp antisense in a molar ratio of 1:5, using T4 DNA ligase. Afterwards, the DNA ligated samples were transformed into *E. coli* αDH5 cells using the heat-shock protocol and the plasmid DNA was extracted and purified by alkaline lysis (section 3.4.1.4.) to screen the *E. coli* transformants containing the correct plasmid construct. The plasmid DNA was digested with SalI (Thermo Scientific) at 37ºC for at least 3 hours and DNA fragments were separated on an 0.8% (w/v) agarose gel electrophoresis. The pSAS39 construction was further validated by nucleotide sequencing as a paid service from MWG Eurofins (Germany), using the primer pBAD_RWD. pSAS39 allows the controlled expression of the lpp antisense sequence by an arabinose inducible BAD promoter sequence upon induction with L-(+)-Arabinose, having the *dhfr* gene encoding to resistance to trimethoprim.

3.3.2. Physiological tests

3.3.2.1. *E. coli* growth curves

In order to evaluate whether the induction of the lpp antisense has any effect on the *E. coli* BL21 (DE3) cell growth, four different cell cultures, two of *E. coli* BL21 (DE3), BL21 (DE3) with pMLBAD and BL21 (DE3) with pSAS39, were monitored by measuring the OD_{600nm}. Primarily, pre-inoculums were prepared in 20 mL of LB liquid medium, incubated overnight at 37 ºC with 250 rpm of orbital agitation. The media for the cells containing the pMLBAD or the pSAS39 plasmids was supplemented with 100 µg/mL of trimethoprim. Afterwards, from these pre-inoculums, 50 mL of LB liquid medium were inoculated at a starting OD_{600nm} of 0.1, containing 100 µg/mL of trimethoprim when required. In order to induce the production of the lpp antisense, arabinose was added to all the cell cultures to a concentration of 2 % (w/v), except one containing *E. coli* BL21 (DE3) cells, to serve as an inducer-free control. The cell cultures were left to grow in the same conditions as before. Samples were taken every 30 minutes and the corresponding OD_{600nm} was measured in a Hitachi U-200 spectrophotometer. When the cell growth reached two hours, further arabinose was added once again (2 % (w/v)), in order to counteract the potential depletion of the previously added inducer. Samples from the cell cultures including the pMLBAD and the pSAS39 plasmids were collected for evaluation of lpp mRNA levels, as described in section 4.1.2.

With the same objective in mind, 4 other independent cultures containing either the pMLBAD or the pSAS39 plasmid were grown with the main difference being the moment of antisense induction. First of all, 2 cell cultures were developed in the same conditions as mentioned above, but the inducer L-(+)-arabinose was added at 3 hours or 4 hours of cellular growth, T3 or T4, respectively. Media samples were also taken at three different moments, for the evaluation of L-(+)-arabinose levels: right before induction (T3 or T4), 30 minutes after induction (T3.5 or T4.5), and 1 hour after induction (T4 or T5).

Another cell culture was developed where the overnight culture was used to inoculate an intermediate culture that was incubated at 37 ºC, 250 rpm, for 2 hours with induction of antisense production in the beginning (T0). Then, the cells were collected at 6000 xg for 10 minutes at 4ºC, and served as inoculum for fresh medium cell cultures, at an initial OD_{600nm} of 0.1. The cultures were left to grow in similar conditions and aliquots of culture broth were harvested for evaluation of L-(+)-arabinose levels.

3.3.2.2. Susceptibility to salts

Three cell cultures, *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) plus pMLBAD and *E. coli* BL21 (DE3) plus pSAS39 were grown each in LB liquid medium, containing 100 µg/mL of trimethoprim when required, at 37 ºC and 250 rpm of orbital agitation for 3 hours. After measuring the final OD_{600nm} values in each culture, the cells are harvested by centrifugation at 9000 rpm (VWR microstar 12) for 3 minutes. The resulting pellets are then resuspended with sterile NaCl 0.9 % (w/v) to a final OD_{600nm} of 1.0. The assays were performed in a 96-wells sterile microplate containing 200 µL of sterile LB liquid medium, where it was possible to infer the susceptibility of the different cultures to a varying concentration of NaCl (10%, 9%, 8%, 7%, 3.5%, 1.75% and 0.875%). The microplate was then incubated overnight at 37ºC, resuspended and the OD_{600nm} was measured using the SPECTROstarNano (BMG LABTECH) microplate reader. The experiment was performed at least three times independently in duplicated wells.

3.3.3. RT-PCR for lpp mRNA levels

3.3.3.1. RNA extraction from culture samples

To analyze lpp mRNA levels in culture samples, at a specific time, the OD_{600nm} of the culture was measured and a sample with total volume following the ratio 2/OD600nm was collected. This sample was then centrifuged at 10000 rpm (Microstar 17, VWR) for 5 minutes at 4 ºC. To storage and stabilize the extracted RNA, 500 µL of RNA later (ambion) was added and the samples were stored at -20 ºC until further use.

Bacterial culture samples were thawed and their RNA was extracted using the RiboPure – Bacteria Kit (ambion), according to the manual instructions. Then, the samples were treated with DNAse I (ambion) following the manufacturer's instructions. The extracted RNA was quantified and a PCR (Polymerase Chain Reaction) experiment was performed to evaluate the contamination with DNA. To amplify the *E. coli* lpp gene by PCR it was used the primers LPP_Upper (5'-AAGGATCCATGAAAGCTACTA -3') and LPP_lower (5'- AACTCGAGCTTGCGGTATTTAG -3') that were designed based on the sequence of the gene encoding the Lpp Braun lipoprotein from *E. coli* BL21 (DE3) and synthesized by STAB VIDA (Portugal). The reaction mix for the amplification contained 100 ng RNA sample, 0.02 U/µL Phusion® High-Fidelity DNA polymerase enzyme (Thermofisher), 1x of Phusion HF buffer (Thermofisher), 200µM of each Deoxynucleotide Triphosphates (dNTPs), 0.5 µM of each primer and distilled water for a total reaction volume of 20 µL. The samples were subjected to an

initial denaturation at 95 ºC for 3 minutes, followed by 30 cycles of denaturation (95 ºC for 45 seconds), annealing (56ºC for 30 seconds) and elongation (72 ºC for 50 seconds) and a final elongation step for 7 minutes at 72 ºC.

The PCR product was visualized on an agarose gel to check if the samples were contaminated with the lpp gene DNA.

3.3.3.2. First strand cDNA synthesis

After confirming the absence of the lpp gene DNA on the sample, the RT-PCR (Real time-polymerase chain reaction) procedure was initiated with the first step being the transformation of RNA into first-strand cDNA, following the protocol from Maxima Scientific Reverse Transcriptase (Thermo Scientific). For this, the reaction components were added according to the protocol established for total RNA sample (1 µg) and gene-specific primer (20 pmol) and the primers used on each sample are displayed on table 2. To be able to infer on the levels of lpp transcript, the 16 S rRNA transcript will be also analyzed, since this RNA must be constantly present in the cells and does not depend of exterior stimulus or induction. The reaction was incubated at 65ºC to allow the synthesis of cDNA from the GC-rich RNA template.

Table 2 – Primers and respective characteristics used for cDNA synthesis from lpp mRNA and the 16S rRNA.

Primer	Sequence	Size (bp)	Melting temperature (°C)
Lw_Lpp-RT	5'-TTT AGC AGC CTG AAC GTC GGA A-3'	22	59
RT16S_1492_RV	5'-GGW TAC CTT GTT ACG ACT T -3'	19	56.9

3.3.3.3. Quantitative PCR reaction (qPCR)

The second step of the RT-PCR procedure consisted on a quantitative PCR experiment. For this, a reaction mix was prepared with the detailed composition being displayed in table 3. The forward (upper) and reverse (lower) primers used are presented in table 4.

Table 3 – Reaction mix composition of quantitative PCR reaction. The quantities and concentrations of each compound are displayed in the table and the volumes are based on a standard 20 µL final reaction mix volume.

NZYSpeed qPCR Green Master Mix (2x), ROX	Forward primer (10 µM)	Reverse primer (10 µM)	cDNA template	Nuclease-free Water
10 µL, 1x	0.8 µL, 400 nM	0.8 µL, 400 nM	100 ng	As required to 20 µL

Table 4 – Primers and respective characteristics used for quantitative PCR analysis of the lpp mRNA and the 16S rRNA transcripts. The forward primer is represented as Up_Lpp-RT and the reverse primer as the Lw_Lpp-RT.

Primer	Sequence	Size (bp)	Melting temperature (°C)
Lw_Lpp-RT	5'-TTT AGC AGC CTG AAC GTC GGA A-3'	22	59
Up_Lpp_RT	5'-TGC TCC AGC AAC GCT AAA ATC G-3'	22	59
RT16S_1114F2 (Denman & McSweeney, 2006)	5'-CGG CAA CGA GCG CAA CCC-3'	18	74.6
RT16S_1275R2 (Denman & McSweeney, 2006)	5'-CCA TTG TAG CAC GTG TGT AGC C-3'	22	66.4

After the mixture is completed and the samples are placed on the tray, the amplification of the cDNA samples can be initiated by thermal cycling RT-PCR. The conditions used are represented in table 5. The experiment was performed in the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems).

Table 5 – Thermal cycling reaction conditions for amplification of cDNA fragments.

Cycles	Temperature	Time	Notes
1	95 ºC	2 min.	Polymerase activation
40	95 ºC 60 ºC	5 seg. 30 seg.	Denaturation Annealing/Extension (Acquiring at end of step)

3.3.3.4. Data analysis procedure of qPCR results

The basis of real-time PCR is a direct positive association between a dye with the number of amplicons (Yuan et al., 2006). The fluorescence signal can be plotted against the cycle number, yielding a linear range at which logarithm of fluorescence signal correlates with the original template amount. A baseline and a threshold can be set for further analysis and the cycle number at the threshold level is defined as Ct number, which is the observed value in most real-time PCR experiments, and the primary statistical metric of interest. For each sample, a target gene and a reference gene for internal control are included for PCR amplification. Firstly, the Ct number is defined and the ΔCt of the sample is calculated as difference between the Ct value of the target gene and the Ct value of the control (Ct_{target} - $Ct_{control}$). Then, the $\Delta\Delta$Ct is calculated as the difference between the ΔCt_{sample} and the $\Delta Ct_{calibrator}$, which is represented by a sample to which all samples are compared to, in this case the pMLBAD culture sample at 2 h after induction (pMLBAD 2 h). Finally, the value for the Relative quantification (RQ) is

reached by determining the value for $2^{-\Delta\Delta Ct}$. This value reflects the fold change compared to the calibrator. For instance, a RQ value of 10 means that the gene is 10 times more expressed in the analyzed sample than in the calibrator sample. Whereas, a RQ of 0.1 corresponds to a gene 10 times less expressed.

3.3.4. Arabinose quantification by HPLC

To evaluate if the added L-(+)-arabinose was being consumed by the cells, the L-(+)-arabinose levels present in the extracellular medium were checked. As mentioned above, L-(+)-arabinose was added at different times of cellular growth in order to induce the production of the antisense molecule. Hence, 1 mL samples of extracellular medium were taken at several timepoints. These samples were centrifuged at 10000 rpm (Sorvall legend 21R) for 5 minutes at room temperature. The supernatant was harvested and a sample with a dilution of 1:2 was prepared, by joining 500 µL of the supernatant with 500 µL of 5 mM H_2SO_4 (mobile phase). The analysis was carried out in a high-performance liquid chromatography (HPLC) method used for arabinose analysis, with a Rezex ROA Organic acid H + 8 % (30 mm x 7.8 mm) column, kept at 65 ºC. Elution was done using 5 mM H_2SO_4, in an isocratic method and a flow rate of 0.5 mL/min. The followed method was based on the method described in Tůma et al. 2020 and quantification of concentration was achieved by using a refraction index (RI) detector. Firstly, a calibration rate was constructed, based on the quantification of arabinose in several arabinose solutions with different concentrations (figure A1 and table A1 of annexes).

3.4. Truncated form of OmpK 21-99 from *Vibrio alginolyticus* ATCC 17749

3.4.1. Construction of a plasmid for overproduction of the truncated form of OmpK 21-99 with signal peptide pelB

3.4.1.1. PCR

The plasmid pJBF2, developed by a previous thesis work in the research group (Lopes, 2017), which contained the *ompK* gene cloned into pet23a+ plasmid, was extracted using the GeneJET Plasmid Miniprep kit (Thermo Scientific). The concentration of plasmid DNA was determined through measurement of the absorbance at 260 nm in a ND-1000 spectrophotometer (Nanodrop, Alfagene), obtaining a concentration of 72.8 ng/mL of plasmid DNA.

The extracted plasmid was used as DNA template for amplification of the nucleotide sequence encoding the OmpK fragment 21-99. For this, oligonucleotide primers were designed based on the sequence of the gene encoding for OmpK from *Vibrio alginolyticus* ATCC 17749 (GenBank: AGV17311.1) and were synthesized by STAB VIDA, as is presented in table 6. The reaction mix for the amplification contained 4 ng plasmid DNA, 0.02 U/µL Phusion® High-Fidelity DNA polymerase enzyme (Thermofisher), 1x of Phusion GC buffer (Thermofisher), 200 µM of each Deoxynucleotide

Triphosphates (dNTPs) and 1 µM of each primer, for a total reaction volume of 20 µL. The samples were subjected to an initial denaturation at 98 ºC for 2 minutes, followed by 30 cycles of denaturation (98 ºC for 10 seconds), annealing (61 ºC for 30 seconds) and elongation (72 ºC for 10 seconds) and a final elongation step for 7 minutes at 72 ºC. PCR amplification was performed in a 2720 thermal cycler (Applied Biosystems).

Table 6 – Indication of the sequence, size and melting temperature of the upper and lower primers used to amplify the truncated form of OmpK 21-99 from *V. alginolyticus* ATCC 17749. Underlined is the restriction enzyme recognition sequence for each primer, targeted by NcoI and XhoI, respectively.

Primer	Sequence	Size (bp)	Melting temperature (°C)
Up-OmpK-P21-99	5'-AAC CAT GGC AGA TTA CTC TG-3'	20	52.3
Lw-OmpK-P21-99	5'-AAC TCG AGC ATA CGT GGA GC-3'	20	57.2

Afterwards, the PCR product was visualized on an agarose gel to check if the fragment presented the expected size of 251 bp.

The resulting band was extracted using the PCR clean-up gel extraction kit (Macherey-Nagel), following the manufacturer's instructions. The amount of DNA purified from the gel was quantified by measuring the absorbance at 260 nm in a ND-1000 spectrophotometer (Nanodrop, Alfagene).

3.4.1.2. Agarose gel electrophoresis

Agarose gel electrophoresis was prepared as described in Maniatis et al. 1982. In order to separate the DNA fragments, gels with 0.8 % (w/v) concentration of agarose were prepared in TAE 1X buffer (dilution from TAE 50X – 242 g/L Tris-base, 57.1 mL/L acetic acid, 37.2 g/L $Na_2EDTA•2H_2O$). Green Safe (NZYTech) was added to the gel at a concentration of 2 µL per 40 mL of agarose, to allow nucleic acids staining. To prepare samples, 3.3 µL of DNA loading die 6X (Thermo Scientific) was added to each 20 µL of DNA sample. In samples, with expected bands lower that 500 bp it was added 0.1 µL of Green Safe. The molecular weight used was GeneRuler 1 kb Plus DNA ladder (Thermo Scientific), at a concentration of 0.5 µg/µL. After placing the plasmid DNA solutions onto the lanes, the gel was run at 100 V until the bromophenol blue line from the DNA loading die reached ¾ of the gel. Once the run was completed, the electrophoresis result was immediately visualized by UV light in a transilluminator (Biorad).

3.4.1.3. Cloning of truncated form 21-99 of OmpK in pET22b(+)

The 251 bp PCR product of truncated form 21-99 of OmpK and the pET22b+ plasmid were digested overnight with the restriction enzymes Xho I and Nco I (Thermo Scientific) according to manual instructions.

The restricted samples went through ethanol precipitation of the DNA, with the objective of purification and concentration of the DNA samples. Therefore, was added to the samples 3 M sodium acetate in the volume ratio of 1:10 (solution:sample), and 2.5X cold 100 % ethanol of the sample volume. The mixtures were incubated at -80 ºC for at least one hour. Then, the samples were centrifuged for 30 minutes at 15000 rpm and 4 ºC (Sigma 2K15). The supernatant was discarded using a syringe and and the DNA pellet was washed with 500 µL of 70 % ethanol (v/v). After discarding the supernatant, the pellet was dried on the speed vaccum for 15 minutes at 45 ºC, using the alcohol dry (V-AL) program (Concentrator plus, Eppendorf). Lastly, the dried pellet was resuspended in 20 µL of H_2Od. The sticky-end 251 bp fragment was ligated into the NcoI/XhoI-digested pET22b+ using T4 DNA ligase (Thermo Scientific) and a molar ratio 1:10 (vector:fragment), yielding pFCN1. Afterwards, the DNA ligated samples were transformed into *E. coli* αDH5 cells using the heat-shock protocol. To screen the colonies for the construction, the plasmid DNA was extracted and purified by alkaline lysis (section 3.4.1.4.), digested with HincII (Thermo Scientific) at 37ºC overnight and DNA fragments were separated on an 0.8 % (w/v) agarose gel electrophoresis. The pFCN1 construction was further validated by nucleotide sequencing as a paid service from MWG Eurofins (Germany). pFCN1 allows the controlled expression of the truncated form 21-99 of OmpK by the T7 promoter upon induction with isopropyl β-D-thiogalactoside (IPTG), having the PelB signal sequence at N-terminal and the 6x His-tag at the C-terminus.

3.4.1.4. Plasmid DNA extraction by alkaline lysis method

The alkaline lysis method was employed in order to extract the plasmid DNA of different cell colonies and later evaluate whether the ligated plasmid or the cloning vector pET-22b(+), were successfully ligated and transformed into the cells. Various cell colonies were picked from the medium plate containing Ap150, each resuspend in 150 µL of solution I (table 7) and left to incubate at room temperature for 5 minutes. Next, 200 µL of solution II (table 7) were added and the samples were mixed by inversion, and left to incubate on ice for 5 minutes. Then, after adding 150 µL of solution III (table 7), the tubes were inverted and placed on ice for at least 10 minutes. After that time, the samples were centrifuged during 10 minutes at maximum speed at room temperature. The resulting supernatants were transferred to new tubes, where 1 mL of cold 100 % ethanol were added. After mixing, the samples were left on ice for at least 10 minutes to allow DNA precipitation, followed by centrifugation for 10 minutes, 15300 rpm (Sigma 2K15) at 4 ºC. The supernatant was discarded with help of a syringe, the pellet was washed with 500 µL of 70 % (v/v) ethanol, and quickly vortexed to improve mixing. Once again, the samples were centrifuged for 5 minutes at 15300 rpm at 4 ºC and the supernatant discarded. The DNA pellets were left to dry by vaccum concentration (Concentrator plus, Eppendorf), at 45 ºC for 15 minutes with the alcohol dry (V-AL) program, resuspended in 30-40 µL of H_2Od and stored at -20 ºC.

Table 7 – Indication of the solutions and respective composition used in the alkaline lysis method.

Solution I (pH 8)	Solution II	Solution III (pH 4.5-5.5)
50 mM Glucose 10 mM EDTA 25 mM Tris-Base 5 mg/mL lysozyme 0.5 µg/mL RNAse I	0.2 M NaOH 1 % (w/v) SDS	3 M potassium acetate 2 M glacial acetic acid

3.4.2. OmpK 21-99 fragment overexpression

3.4.2.1. Optimization of the OmpK 21-99 fragment overexpression in *E. coli* BL21 (DE3)

Several different conditions were performed in order to achieve optimization of the OmpK 21-99 fragment overexpression in *E. coli* BL21 (DE3) cells. The followed strategy was based on overexpressing the OmpK fragment under different temperatures (37 ºC, 30 ºC, 25 ºC and 18 ºC), to see if by changing this condition from higher temperatures to lower temperatures, it would reflect on peptide production. For this, a preinoculum was prepared using 30 mL of LB liquid medium, containing the *E. coli* BL21 (DE3) with the pFCN1 plasmid, supplemented with ampicillin (150 µg/mL). The preinoculum was initially incubated at 37 ºC with orbital agitation of 250 rpm, until it reached an OD_{640nm} higher than 1. Then, the same preinoculum was used to inoculate 100 mL of SB liquid medium containing ampicillin 150 µg/mL, at an initial OD_{640nm} of 0.1 and left to incubate in the same conditions. Once the cell culture reached an OD_{640nm} between 0.6 and 0.8, the overexpression was started by inducing the T7 promoter with addition of 0.4 mM of IPTG. Afterwards, the cell culture was incubated at the designed temperature for overexpression (37 ºC, 30 ºC, 25 ºC or 18 ºC), and 250 rpm, while samples were taken after every hour after induction (until T4 for temperatures 37 ºC and 30 ºC). With the objective of improving the yield of soluble protein production, overexpression at low temperatures can be a good strategy. The inoculation procedure was similar to what is explained above, but after induction the cultures are grown at 25 ºC with culture samples being taken every hour until T3, or at 18 ºC with samples taken at the moment of induction and after 16 hours. As before, in the end, the cells were collected by centrifugation and the pellet stored at -80 ºC. In the end, the cell cultures were centrifuged at 7000 g for 5 minutes at 4 ºC, and the pellet was saved at -80 ºC for further tests. The samples containing the protein extracts were then treated and used to check the overexpression process by carrying out an SDS-PAGE. Table 8 presents a summary of the procedures explained above.

Electrocompetent *E. coli* BL21 (DE3) cells were transformed by electroporation (section 3.2.2.) with the cloning vector pET-22b(+) without the OmpK fragment sequence. The overexpression was carried out at 30 ºC in a similar way as explained above. This strategy was carried out with the objective of understanding whether there was some protein being highly expressed in the control (pET22b+) in the same MW of the expected band of OmpK peptide overexpression (pFCN1).

Table 8 – Summary of the conditions and crucial steps in overexpression of the OmpK 21-99 fragment.

Cell culture	Condition	Induction	Overexpression	Harvested samples
***E. coli* BL21 (DE3) with pFCN1 plasmid**	37 ºC	0.4 mM IPTG $0.6 \leq OD_{640} \leq 0.8$	At specific temperature 250 rpm	T0, T1, T2, T3 and T4
	30 ºC			
	25 ºC			T0, T1, T2 and T3
	18 ºC			T0 and T16
***E. coli* BL21 (DE3) with pET-22b(+) plasmid**	30 ºC			T0, T1, T2, T3 and T4

3.4.2.2. Overexpression using *E. coli* BL21 (DE3) pSAS39 cells

Electrocompetent *E. coli* BL21 (DE3) cells with the pSAS39 plasmid were transformed by electroporation with the pFCN1 plasmid containing the OmpK fragment 21-99, following the protocols previously explained (section 3.2.2.). This strategy could provide a better understanding of the effect the antisense was having on the permeabilization of the cell wall, that way improving the exportation of the overexpressed peptide to the extracellular medium. The overexpression was carried out in similar conditions as explained above (section 3.4.2.1.), with the difference that the cell cultures were additionally supplemented with trimethoprim 100 µg/mL, to enable selection of the cells containing the pSAS39 plasmid. 2% L-(+)-arabinose was added 3 hours (T3) after the beginning of overexpression, to induce the antisense production. A control culture was carried out, without addition of L-(+)-arabinose to compare the presence of the OmpK peptide in the extracellular medium. As before, samples were taken during the experiment and the cells were harvested when the overexpression reached 4 hours.

3.4.2.4. Periplasmic fraction isolation by osmotic shock

In order to evaluate if the peptide OmpK fragment 21-99 was successfully transferred to the periplasm, isolation of the periplasmic fraction was performed. The periplasmic fraction preparation involved an osmotic shock protocol described in the pET manual (Novagen). Briefly, after stopping peptide overexpression, the whole culture is centrifugated at 6000 xg for 5 minutes at 4 ºC. The medium fraction obtained is either discarded or saved for further analysis. The obtained pellet, which contains the cell fraction, was resuspended in 30 mL of 30 mM Tris-HCl, 20 % (w/v) sucrose, pH 8. EDTA pH 8 was added to a final concentration of 1 mM and the suspension was slowly stirred at room temperature for 10 minutes. Afterwards, the suspension was centrifugated at 10000 xg for 10 minutes at 4 ºC and the obtained supernatant was discarded. Next, the obtained pellet was resuspended in 30 mL of ice-cold 5 mM $MgSO_4$ and the cell suspension was stirred slowly for 10 minutes on ice. During this step, the periplasmic proteins are being released into the buffer. Once again, the suspension was centrifuged in similar conditions as before. Ideally, in the end, the pellet contains the shocked cells and the supernatant the soluble proteins from the *E. coli* periplasm. The totality of the supernatant containing

the periplasmic fraction was concentrated by spin filtration using an Amicon Ultra-4 (Merck) with a molecular weight cut-off (MWCO) of 3 kDa. The concentration was performed by successive centrifugations at 7500 xg for 20 minutes and 4 ºC, in a fixed angle rotor, obtaining in the end a solution concentrated around 100X. The samples were mixed with gel loading buffer in a ratio of 1:4 (gel loading buffer:periplasmic fraction sample).

3.4.2.5. SDS-PAGE (denaturing polyacrylamide gel electrophoresis)

SDS-PAGE are used to separate proteins according to their molecular weight. In this work, the gels were prepared following the compositions observed in table 9. The running gel presented a concentration of 15 % (w/v) in acrylamide, better for separation of smaller MW proteins.

Table 9 – Composition and respective concentrations of the SDS-PAGE.

Stock Solution	Running gel 15 % (w/v)	Stacking gel 4 % (w/v)
Running buffer (1.5 M Tris base, pH 8.8)	1.875 mL	-
Stacking buffer (0.5 M Tris base, pH 6.8)	-	375 µL
Acrylamide stock (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)	2.5 mL	270 µL
H_2Od	525 µL	1.305 mL
SDS 10 % (w/v)	50 µL	20 µL
APS 10 % (w/v)	50 µL	30 µL
TEMED	2.5 µL	2 µL

The mixture for each type of gel was prepared until the addition of ammonium persulphate (APS) 10 % (w/v) and N,N,N',N'-tetramethylethylenediamine (TEMED), which were only added once the mixture was about to be poured into the gel structure to prevent premature polymerization of the acrylamide. Once the polymerization was completed, the gel was immersed in running buffer 1X (running buffer 10 X: 0.25 M Tris base, 1.92 M glycine, 1% (w/v) SDS, pH 8).

As mentioned before, cell samples were harvested during overexpression, following a ratio of $0.6/OD_{640nm}$. The samples were centrifuged at 13500 rpm (MicroStar 12 VWR), during 5 minutes at room temperature. After discarding the supernatant, the cell pellet was resuspended in 40 µL of gel loading buffer (100 mM Tris base pH 6.8, 4 % (w/v) SDS, 20 % (w/v) glycerol, 0.2 % (w/v) bromophenol blue and 200 mM DTT), followed by boiling at 95 ºC for 5 minutes. For each lane, 5 µL of the molecular weight marker (PageRuler Unstained Broad Range Protein Ladder; Thermo Scientific; 5-250 kDa range), or 10 µL of protein samples were applied in the corresponding well. To enable protein separation according to the molecular mass, a voltage of 170 was applied until the bromophenol blue present in the loading buffer reached the bottom of the gel. Then, the polyacrylamide gel was stained by emersion

in BlueSafe staining solution (NZYTech) for at least 1 hour until overnight, followed by washing with distilled water.

3.4.2.6. Detection of produced His-tagged proteins by Western Blot

For the Western Blot experiment, an SDS-PAGE gel is prepared as presented above. Once the run is complete, the polyacrylamide gel is immersed in 30 mL of transfer buffer (Bjerrum Schafer-Nielsen buffer: 48 mM Tris base, 39 mM glycine, 20 % (v/v) methanol, 0.04 % (w/v) SDS pH 9), for at least 15 minutes. At the same time, a nitrocellulose (NC) membrane (PALL Corporation) was immersed in the transfer buffer for 5 minutes and 3MM Whatmann filter papers were quickly immersed in the same buffer. Then, the system was assembled as shown in figure 7.

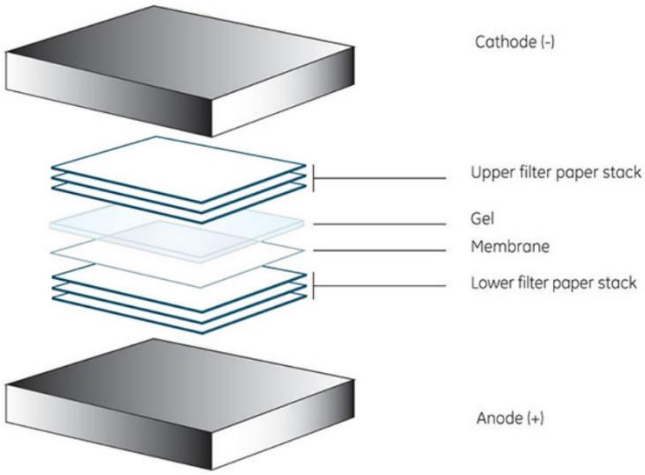


Figure 7 – Construction of the gel-membrane sandwich in the Western-blot system. All components are soaked in transfer buffer and placed in direct contact with the electrodes, compressing the sandwich (adapted from Cytiva Life Sciences, 2021).

After assembling the system, transfer of proteins to the NC membrane was performed at 15 V and 120 mA for 50 minutes in a semi-dry electrophoretic transfer unit (Trans-Blot SD, BioRad). After protein transfer is completed, the membrane is left to incubate in 40 mL of blocking buffer (5 % (w/v) skim milk in PBS 1X), with gentle agitation at 4 ºC overnight or for 1 hour at room temperature. Sometimes, before this step, an optional staining step can be included where the membrane is incubated with Ponceau (NZYTech) in order to visualize whether the proteins were successfully transferred, followed by a few washing steps with H_2O_d to remove the staining solution from the membrane. In this work, this step was only included in one experiment. After incubation with the blocking buffer, the membrane was washed three times for 5 minutes each with 30 mL of washing buffer (0.05 % (v/v) Tween 20 in PBS 1X). Afterwards, the membrane was incubated at room temperature with agitation in 20 mL of antibody solution (washing buffer containing 1 % (w/v) BSA and 1:2000 dilution of monoclonal anti-polyhistidine peroxidase conjugate antibody (SIGMA)), for 2 hours. Once again, the membrane was washed 3 times as previously described. The next step included membrane incubation for 3 minutes in the dark with 2 mL of enhanced chemiluminescence (ECL) detection reagent (composed of two solutions; solution 1: 2.5 mM luminol, 400 µM p-coumaric acid, 100 mM Tris-HCl pH 8; solution 2: 0.15

% (v/v) H_2O_2, 100 mM Tris-HCl pH 8; being the final solution prepared by mixing both solutions in a ratio 1:1). After removing the excess of reagent, detection of target His-tagged protein was performed using the cheluminescence imaging system Fusion Solo (Vilber Lourmat).

4. Results and Discussion

4.1. Analysis of *E. coli* BL21 (DE3) outer membrane permeabilization using lpp antisense technology

4.1.1. Analysis of *E. coli* growth curves under lpp antisense expression

4.1.1.1. *E. coli* BL21 (DE3) growth curves

The monitorization of the cells' growth was conducted in order to observe whether the behaviour of the cells was impacted by the expression of the lpp antisense. For this, the cells (*E. coli* BL21 (DE3), *E. coli* BL21 (DE3) with pMLBAD and *E. coli* BL21 (DE3) with pSAS39), were grown in the presence of L-arabinose 2 % (w/v) functioning as an inducer of the pBAD promoter, hence being responsible for the production of the lpp antisense sequence. Additionally, a control assay was also performed with the *E. coli* BL21 (DE3) cells in the absence of the inducer. The growth curves were followed by measuring the OD_{600nm} values every 30 minutes until 6 h growth (figure A2.1. in Annexes A2). Afterwards, the specific growth rates were determined for each culture, and the final OD_{600nm} values were registered and are represented in table 10.

Table 10 – Specific growth rates (h^{-1}) and final OD_{600nm} values for *E. coli* BL21 (DE3), with and without induction, *E. coli* BL21 (DE3) with pMLBAD and *E. coli* BL21 (DE3) with pSAS39 cell cultures, grown in LB liquid medium at 37 ºC and 250 rpm. (I) – Indicates that cells were induced with L-arabinose 2 % (w/v).

Strains	Specific growth rates (h^{-1})	Final OD_{600nm}
***E. coli* BL21 (DE3)**	0.991	3.204
***E. coli* BL21 (DE3) (I)**	0.981	2.470
***E. coli* BL21 pMLBAD (I)**	0.897	2.650
***E. coli* BL21 pSAS39 (I)**	0.860	2.280

As it is possible to observe from the values of specific growth rates in table 10, the cells corresponding to the wild type strain had a slightly higher growth when compared to the other cells. On the other hand, the cells containing the pSAS39 plasmid, that contains the antisense lpp, are the cells with the slowest growth, reflected by the lowest specific growth rate (0.860 h^{-1}). These observations are due to the fact that the cells that contain the pMLBAD or the pSAS39 plasmid need to make a higher effort during the growth than the wild type cells, suggesting that the presence of the plasmid affects the

growth of the cells. The difference between both cultures is not significative to be explained by the effect of the lpp antisense on the cells. Lastly, the cell culture containing *E. coli* BL21 (DE3) in the absence of L-arabinose allowed to infer on the possible effect of this sugar on the growth, since its presence at such a high concentration (2 %) can cause stress to the cells, explained by the difference of behaviors in final biomass achieved of the wild-type cells in different culture environmental conditions.

Furthermore, other 3 additional growth curves with different conditions established were performed, to which the logarithmic growth curves are represented in figure A2.2. in Annexes. The obtained specific growth rates are showed below in table 11. Condition A corresponds to the cell culture that apart from the overnight inoculum, an intermediate inoculum was also prepared and was used to inoculate the final cell culture. This intermediate culture was induced at the T0 timepoint with 2 % L-Arabinose and left to incubate at 37 ºC and 250 rpm for 2 h. Then, the final culture was incubated in the same conditions and the growth was followed for 3.5 h, with medium samples being taken every 30 minutes. Conditions B and C are the growth curves with induction either at 3 hours (T3) or 4 hours (T4), respectively. In those cases, medium samples were harvested at the time of induction, 30 minutes and 1 hour after induction. Globally, in every experiment, the cell culture containing the antisense sequence (pSAS39) did not grow as much as the control culture without the antisense (pMLBAD), which might indicate that the antisense is influencing cell growth by preventing the formation of the Lpp protein, or that once more, the added arabinose is preventing the cells from growing as they normally would.

Table 11 – Specific growth rates (h^{-1}) for *E. coli* BL21 (DE3), in different conditions. The cell cultures performed either contain the cloning vector pMLBAD or the antisense expression plasmid pSAS39, grown in LB liquid medium at 37 ºC and 250 rpm. Cells were induced by addition of 2 % L-arabinose (w/v). Conditions (A) – Culture process included an intermediate culture induced at T0, used to inoculate the final culture. Induction at T0 and T2; (B) – Induction with arabinose at T3. (C) – Induction with arabinose at T4.

Conditions	Strains	Specific growth rates (h^{-1})	Final OD_{600nm}
A	*E. coli* BL21 pMLBAD	0.910	0.867
	E. coli BL21 pSAS39	0.854	0.095
B	*E. coli* BL21 pMLBAD	1.008	0.920
	E. coli BL21 pSAS39	0.992	0.924
C	*E. coli* BL21 pMLBAD	1.024	1.095
	E. coli BL21 pSAS39	0.999	1.089

4.1.2. RT-PCR for lpp mRNA levels

Quantitative PCR measures gene expression, more specifically how much of a specific mRNA exists in the tested samples. A small region of the mRNA target is amplified with oligonucleotides and a fluorescence probe. The detector measures the intensity of fluorescence emitted by the probe at each cycle, and at each PCR cycle the number of PCR products doubles and the fluorescence builds up. Typically, the qPCR curve has an exponential phase followed by a plateau phase. The Ct (cycle threshold) measure is a specific PCR cycle and represents the basic result of a qPCR experience. This parameter is measured in the spot where the PCR curve crosses a threshold line (similar for samples tested with the same gene), placed in the linear phase.

Most of the cases, including in this work, the qPCR experiment will give a “relative expression” which is a variation of the expression of a given gene between two samples. An endogenous control is necessary to guarantee the success of the experiment and it is typically a gene that does not vary between all of the samples tested, in this work that gene was the 16S rRNA. A calibrator was also defined as the sample that all others are compared to and to which the RQ value is equal to 1, because it does not vary compared to itself. The Ct value is the one used for analysis meaning that the higher the Ct, the less the mRNA detected, since more cycles of amplification would be necessary to detect fluorescence. For highly expressed genes, the Ct value is smaller. To reach the final value, a series of calculations were performed.

The obtained RQ values calculated for each cell culture analyzed, as explained in section 3.3.3.3., are represented in figure 8. The values were determined in relation to the pMLBAD 2 h culture, which was used as the calibrator and is given a RQ equal to 1.

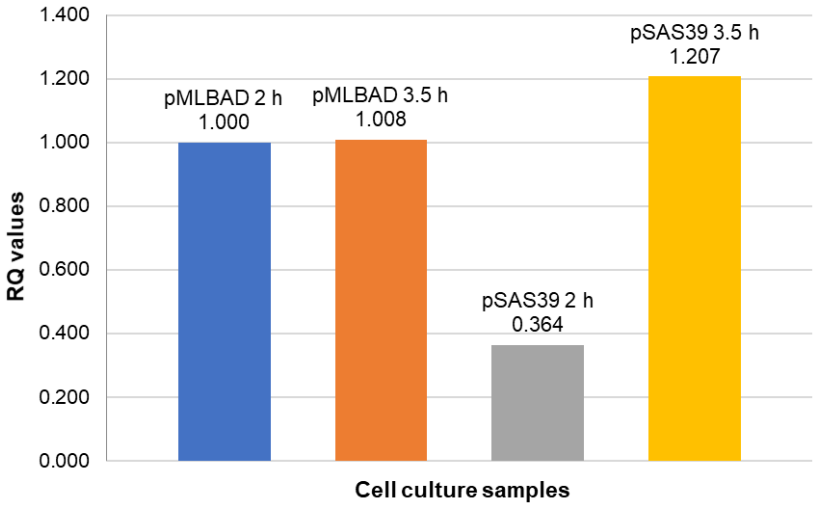


Figure 8 – RQ values representing the expression values of the lpp mRNA for the different cultures being tested. Blue: sample from the pMLBAD culture taken at 2h growth induced at T0. Orange: sample from the pMLBAD culture taken at 3.5h growth induced at T0 and T2. Grey: sample from the pSAS39 culture taken at 2h growth induced at T0. Yellow: sample from the pSAS39 culture taken at 3.5h growth induced at T0 and T2.

The RQ values give an evaluation of the expression levels of the Lpp transcript. As it is possible to infer from figure 8, there is a reduction in the expression of the Lpp transcript in the cell culture containing the antisense, 2 h after induction at the T0 timepoint (pSAS39 2 h), when comparing to the control cell culture without the antisense (pMLBAD 2 h). However, at 1.5 h after the second induction at the T2 timepoint, the effect appears to disappear and the transcript levels increase to the normality. It has been shown before that in the case of the $araP_{BAD}$ promoter, the production of the target protein can be greatly increased by inducing with different sub-maximal concentrations of arabinose (Guzman, Weiss, & Beckwith, 1995). However, it has been reported that protein expression varies depending on the amount of active sugar permeases in each cell. This means that while the final protein yield can be manipulated, the amount of protein per cell is variable, with certain cells producing high quantities of the target protein and other cells not producing any protein at all (Doherty et al., 1993; Dong et al., 1995). In the case of production of toxic products, as can be the case of the lpp antisense due to the inhibiting effect it has on the production of the Lpp Braun lipoprotein, the subpopulation of cells with elevated synthesis may perish and the effect of the antisense is lost. Khlebnikov et al. 2000 demonstrated that to overcome the all-or-none induction demonstrated by the arabinose-inducible promoter P_{BAD}, the inclusion of a transport gene that is controlled independently of the inducer (for example with isopropyl-β-D-thiogalactopyranoside, IPTG), is quite important in achieving regulatable and consistent induction in all cells of the culture, otherwise the native transporter gene will be controlled by the arabinose-inducible promoter, giving rise to subpopulations of cells that are fully induced and uninduced.

4.1.3. Arabinose quantification in *E. coli* antisense induction by HPLC

The calibration rate (observed in table A1 and figure A1 of Annexes), enabled the determination of arabinose concentration in the culture medium over time. *E. coli* BL21 (DE3) have been demonstrated to consume arabinose through its native metabolism (Schleif, 2010), hence this experiment will help to evaluate if the amount of the inducer stays the same or decreases. A_x corresponds to the area under the peaks belonging to arabinose and c_y to the calculated concentration. Table 12 contains the concentration profile of the *E. coli* growth with two moments of induction (T0 and T2), and in which an intermediate culture was included, with the objective of analyzing if the cells would behave differently since they were already induced. By observing the data in table 12, the cell culture without the antisense (pMLBAD) does not present a consumption of the arabinose present in the medium, and confirms the new addition of the inducer at the T2 since the concentration increases almost to the double, as it is possible to see at 2.5 h of growth. Regarding the pSAS39 culture, arabinose seems to be consumed since a slight decrease on the concentration is observable. The values in red in table 12 represent values that don't follow the gradually decreasing tendency the rest of the values demonstrate. Because this assay was only performed once, there is no way of knowing if they correspond to actual measures or to technical errors that might have occurred.

Table 12 – Calculated arabinose concentrations for the samples taken at the several timepoints of *E. coli* BL21 (DE3) growth with the pMLBAD or the pSAS39 plasmid. Cell culture that included an intermediate inoculum (with T0 induction with 2 % L-arabinose and incubated at 37 ºC and 250 rpm for 2 h), used to inoculate the final cell culture. The final cell culture was incubated in similar conditions and the growth was followed for 3.5 h. (I) represents the moments of induction with arabinose. Ax is the intensity of the arabinose IR peaks. Cy is the concentration of arabinose in the culture media.

	pMLBAD		**pSAS39**	
	A_x (AU)	c_y (g/L)	A_x (AU)	c_y (g/L)
T0 (I)	2.70 x 10^6	26.5	2.80 x 10^6	27.1
T0,5	2.90 x 10^6	27.7	2.78 x 10^6	27.0
T1	1.38 x 10^6	19.2	2.74 x 10^6	26.8
T1,5	2.78 x 10^6	27.0	1.01 x 10^6	17.1
T2 (I)	2.55 x 10^6	25.7	2.63 x 10^6	26.1
T2,5	5.27 x 10^6	40.9	5.12 x 10^6	40.1
T3	5.18 x 10^6	40.4	5.11 x 10^6	40.0
T3,5	5.09 x 10^6	39.9	5.00 x 10^6	39.4

AU – arbitrary units of peaks area

Observing the concentration values in table 13, of the cell culture with induction at 3 hour growth (T3), the concentration of arabinose in the culture media does not allow to infer on the consumption by the cell metabolism, since in the case of the cells with the pSAS39 plasmid, the concentration remains stable and with the pMLBAD plasmid, concentration decreases but not gradually, making this assay inconclusive.

Table 13 – Calculated arabinose concentrations for the samples taken at the several timepoints of *E. coli* BL21 (DE3) growth with the pMLBAD or the pSAS39 plasmid. Cell culture incubated at 37 ºC and 250 rpm with induction at the T3 timepoint with 2 % L-arabinose. Culture samples were taken at the moment of induction (T3), 30 minutes (T3.5) and 1 hour (T4) after induction. (I) represents the moments of induction with arabinose. Ax is the intensity of the arabinose IR peaks. Cy is the concentration of arabinose in the culture media.

	pMLBAD		**pSAS39**	
	A_x (AU)	c_y (g/L)	A_x (AU)	c_y (g/L)
T3 (I)	2.99 x 10^6	28.2	2.90 x 10^6	27.6
T3.5	2.69 x 10^6	26.5	2.87 x 10^6	27.5
T4	2.83 x 10^6	27.3	2.91 x 10^6	27.7

AU – arbitrary units of peaks area

In the following study, represented in table 14, induction was carried out at 4 hours of growth. The cell culture with the pSAS39 plasmid shows a slight arabinose consumption, although not considerate. For the pMLBAD culture, the opposite occurs and arabinose concentration rises, indicating a possible technical error occurred during the experiment.

Table 14 – Calculated arabinose concentrations for the samples taken at the several timepoints of *E. coli* BL21 (DE3) growth with the pMLBAD or the pSAS39 plasmid. Cell culture incubated at 37 ºC and 250 rpm with induction at the T4 timepoint with 2 % L-arabinose. Culture samples were taken at the moment of induction (T4), 30 minutes (T4.5) and 1 hour (T5) after induction. (I) represents the moments of induction with arabinose. Ax is the intensity of the arabinose IR peaks. Cy is the concentration of arabinose in the culture media.

	pMLBAD		**pSAS39**	
	A_x (AU)	c_y (g/L)	A_x (AU)	c_y (g/L)
T4 (I)	2.82 x 10^6	27.2	3.09 x 10^6	28.7
T4,5	2.85 x 10^6	27.4	3.04 x 10^6	28.5
T5	2.97 x 10^6	28.1	2.87 x 10^6	27.5

AU – arbitrary units of peaks area

4.1.4. Susceptibility to NaCl

The objective behind testing the cells susceptibility to osmotic stress using sodium chloride (NaCl) was to observe whether the lpp antisense sequence had any influence on the permeability of the cell envelope. If the cell envelope was affected by the antisense, then the cell permeability would increase, allowing a higher salt uptake and a faster loss of viable cells. Figure 9 shows the values obtained for the OD_{600nm} for the different cell cultures, in the presence of several NaCl concentrations (10%, 9%, 8%, 7%, 3.5%, 1.75% and 0.875%).

From the results shown in figure 9, some differences between the wild type strain and the strain containing the antisense are readily observed, with the latter being slightly more susceptible to salts. Although it is still necessary to repeat the procedure and test more stress concentrations, it is possible to hypothesize that the lpp antisense sequence is causing an inhibition effect on the expression of the Lpp membrane protein, as it was expected. At lower concentrations of NaCl, the control culture without the plasmid shows a higher growth, while at higher concentrations the growth impact is very similar between the different cultures.

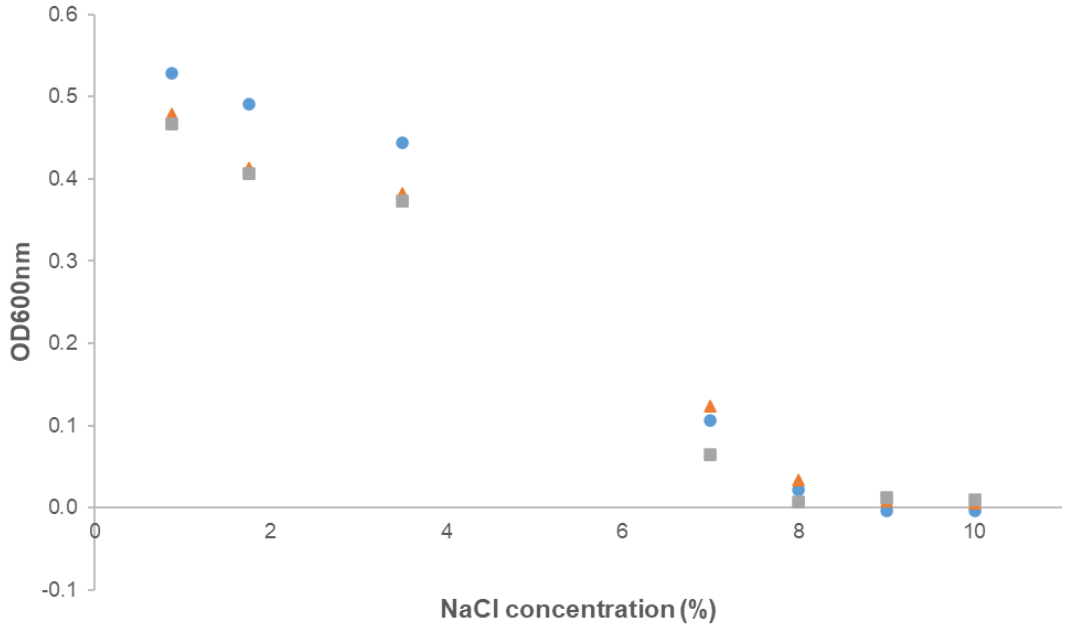


Figure 9 – Final values of OD_{600nm} for *E. coli* BL21 (DE3) (blue), *E. coli* BL21 (DE3) with pMLBAD (orange) and *E. coli* BL21 (DE3) with pSAS39 (gray) cell cultures, grown in LB liquid medium with varying concentrations of NaCl and induced with L-arabinose 2 %, after overnight microplate incubation at 37 ºC.

4.2. Truncated form of OmpK 21-99 from *Vibrio alginolyticus* ATCC 17749

4.2.1. Construction of a plasmid for overproduction of the truncated form of OmpK 21-99

A new plasmid construct was developed in order to obtain the OmpK fragment 21-99 included in the pET-22b(+) vector from Novagen. This vector carries an N-terminal *pelB* signal sequence that directs the protein to the bacterial periplasm, and also contains a C-terminal His-Tag sequence. This would allow an improvement on the overproduction and purification of the peptide, since the peptide would be sent to the periplasm.

As mentioned before, a 0.8 % agarose gel was prepared in order to evaluate if the desired OmpK fragment was successfully amplified in the PCR process, whose conditions are described in section 3.4.1.2. Figure 10 (Left) demonstrates the first attempt at producing the DNA fragment, which was conducted to understand which buffer would work better. Buffers HF and GC conditions were tested and buffer GC condition gave the higher amount of DNA fragment amplified, as it is possible to confirm by the more intense band in the electrophoresis gel, located at the expected PCR product size of 251 bp. In figure 10 (Right), are presented the results from a new PCR amplification of the same fragment, now only using the GC Phusion buffer, which was repeated 4 times in order to end up with a bigger amount of DNA fragment. Once more, the product is in the range of the expected size.

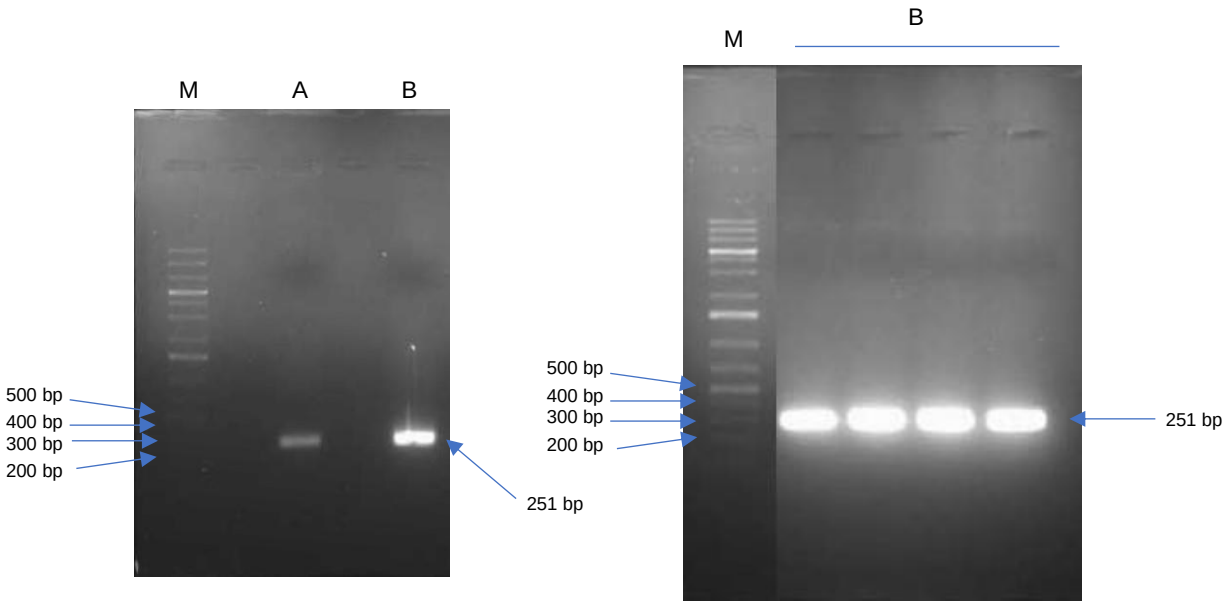


Figure 10 – 0.8 % Agarose gel with the *OmpK* 21-99 DNA fragment amplified by PCR. Left – M: GeneRuler 1 kb Plus DNA Ladder; A: product of PCR batch to amplify the OmpK fragment 21-99, using the 5x Phusion HF buffer; B: product of PCR batch to amplify the *OmpK* fragment 21-99, using the 5x Phusion GC buffer. Right – M: GeneRuler 1 kb Plus DNA Ladder; B: products of four PCR batches to amplify the *OmpK* fragment 21-99, using the 5x Phusion GC buffer.

After amplification, the obtained fragments were digested with NcoI and XhoI and ligated to the pET-22b(+) vector in order to obtain the pFCN1 plasmid. The nucleotide sequence of the recombinant plasmid pFCN1 and the one from pET-22b(+) were used to draw the restriction profile with NEBcutter 2.0 and are demonstrated in figure 11.

pET-22b(+)

#	Enzyme	Specificity	Cuts	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	HincII	GTY▾RAC	2	list	*181, *1620
2	NcoI	C▾CATG_G	1	list	220/224
3	XhoI	C▾TCGA_G	1	list	*158/162

pFCN1

#	Enzyme	Specificity	Cuts	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	HincII	GTY▾RAC	1	list	*1803
2	NcoI	C▾CATG_G	1	list	403/407
3	XhoI	C▾TCGA_G	1	list	*158/162

Figure 11 – Restriction profile of the expression vector used (pET-22b(+)) and the vector constructed (pFCN1). Up – pET-22b(+); Down – pFCN1 (pET-22b(+) + OmpK 21-99); representation of restriction sites for the used enzymes (XhoI, NcoI and HincII).

By comparing the restriction profiles of both the pFCN1 and pET-22b(+) plasmids, a difference in the cut sites is evident. The pET-22b(+) plasmid vector has an additional Hinc II restriction site between the restriction sites for the Xho I and Nco I enzymes, which makes this enzyme a good candidate to distinguish between the recombinant plasmid and the original vector. The pET-22b(+) vector containing the *OmpK* 21-99 fragment was confirmed by restriction with Hinc II (figure 12). For comparison of the size of fragments obtained with digestion, the pET-22b(+) vector was also digested with Hinc II. It was possible to observe in the gel, that the lane corresponding to the pET-22b(+) vector shows two bands (one around 4000 bp and another around 1500 bp), which correspond to the expected fragments obtained after digestion with Hinc II. In this case, the enzyme will cut in two different places, obtaining a fragment of 4054 nucleotides and one fragment of 1439 nucleotides. In the case of the construct, the enzyme Hinc II will only cut the plasmid in one fragment, since one of the previously observed cut sites has been lost after the cloning process. Consequently, only one fragment of 5676 nucleotides can be observed in the agarose gel, which is consistent with the band present in lanes 3 and 4 (figure 12). Therefore, it was possible to conclude that the pFCN1 plasmid was successfully constructed. The plasmid construct was sent to sequencing and the result allowed the confirmation of the success of the cloning process.

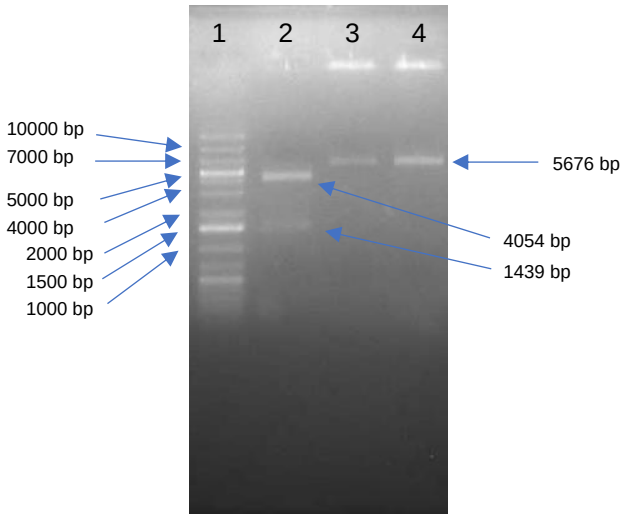


Figure 12 – 0.8 % Agarose gel of the HincII restriction samples of the pFCN1 plasmid transformants and of the pET-22b(+). Lane 1: GeneRuler 1 kb Plus DNA Ladder; Lane 2: pET-22b(+) vector cut with Hinc II; Lane 3 and 4: plasmid extracted from selected transformant cut with Hinc II.

4.2.2. OmpK 21-99 overexpression optimization

In order to understand what strategy would be best to obtain a better overexpression of the OmpK peptide, different approaches were tried out. Since the first experiments taken during the studies of the antisense effect on the cell were conducted at 37 ºC, an initial overexpression at this temperature was developed. Then, overexpression experiments at 30 ºC, 25 ºC and 18 ºC were also developed in order to see if the production of the small peptide would increase by lowering the temperature. Additionally,

an experiment using the BL21 (DE3) cells containing both the pSAS39 plasmid for antisense production and the pFCN1 plasmid for OmpK peptide production was carried out at 30 ºC.

4.2.2.1. Overexpression at 37 ºC

E. coli BL21 (DE3) cells containing the pFCN1 plasmid were grown at 37 ºC and protein production was started after adding 0.4 mM of IPTG, once the cells reached the stage of 0.6-0.8 OD_{640nm}. Figure 13 shows an SDS-PAGE containing total cell fraction samples, taken every hour after induction.

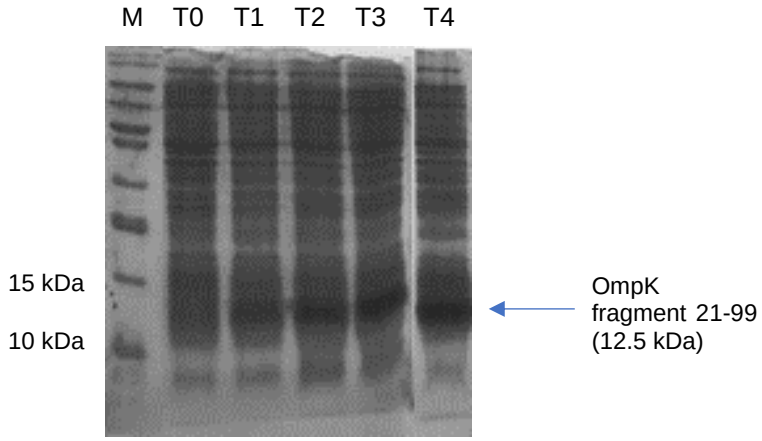


Figure 13 – SDS-PAGE containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 37 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 to T4: protein samples taken during overexpression 0, 1, 2, 3 and 4 hours after induction, maintaining an OD_{600nm} of 0.6.

The OmpK fragment produced has an expected size of 12.5 kDa. Consequently, the result of the overexpression should be observed in the area between the 15 kDa and 10 kDa bands of the protein marker. As it is possible to see, a clear evolution in the production of the peptide is noticeable, since the bands grow in intensity from the samples T1 to the T4. In order to confirm these results, a Western-Blot analysis was conducted to see if the observed bands confirm the presence of the His-tagged OmpK fragment overexpressed.

Figure 14 shows the Western-Blot obtained with the protein samples taken during overexpression at 37 ºC. Since only the overexpressed proteins with a His-tag should be seen on the Western-Blot membrane, as is the case of the desired peptide, this experiment would allow to obtain a more correct analysis of the overexpression experiment. Consequently, the Western-Blot showed low intensity bands corresponding to the expected MW of 12,5 kDa, meaning that the overexpression experiment was not optimal in producing high amounts of the OmpK fragment 21-99, possibly due to the fact that the temperature used might not be the best to produce small peptides in large quantities. For this reason, other overexpression conditions were tested.

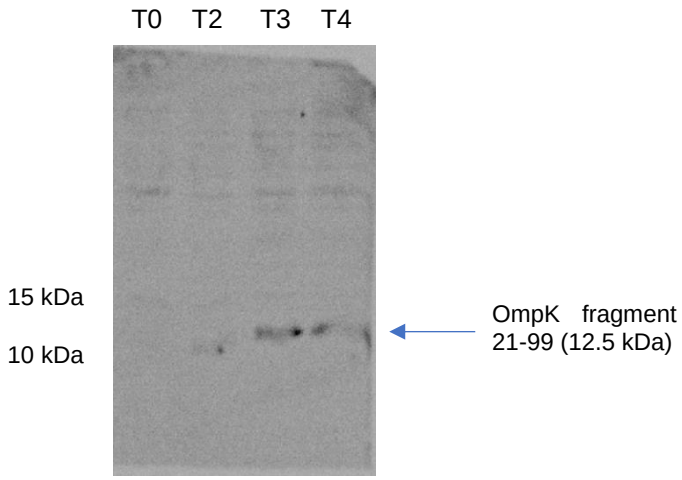


Figure 14 – Western-Blot containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 37 ºC. Lane T0: protein sample taken right before induction (T0); Lanes T2 to T4: protein samples taken during overexpression 2, 3 and 4 hours after induction.

4.2.2.2. Overexpression at 30 ºC

Similar to what is explained above, overexpression of the OmpK fragment 21-99 at 30 ºC was also conducted in order to understand if this condition could be used to express high amounts of the desired peptide. Figure 15 shows the SDS-PAGE electrophoresis gel obtained containing the protein samples taken during the experiment (T0 to T4).

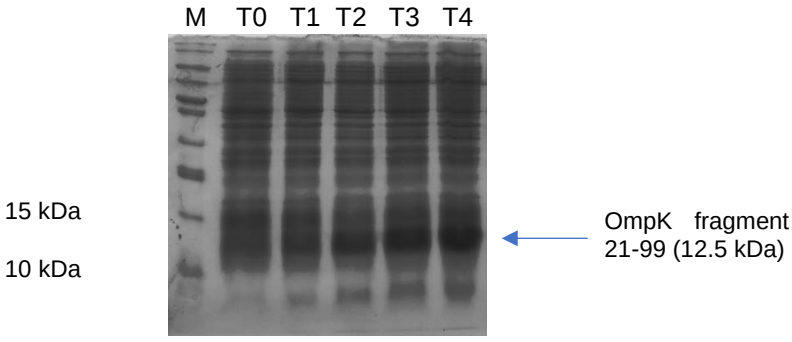


Figure 15 – SDS-PAGE containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 30 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 to T4: protein samples taken during overexpression 0, 1, 2, 3 and 4 hours after induction, maintaining an OD_{600nm} of 0.6.

Once again, this strategy resulted in a visible overexpression of the peptide, since it is possible to observe a clear increase in the intensity of the bands from the sample T1 to the T4 in the area between 10 kDa and 15 kDa, meaning that the overexpression increased with the increase of cell growth. To make sure that the protein fraction being produced corresponds to the desired peptide, a Western-Blot experiment was conducted, which can be observed in figure 16.

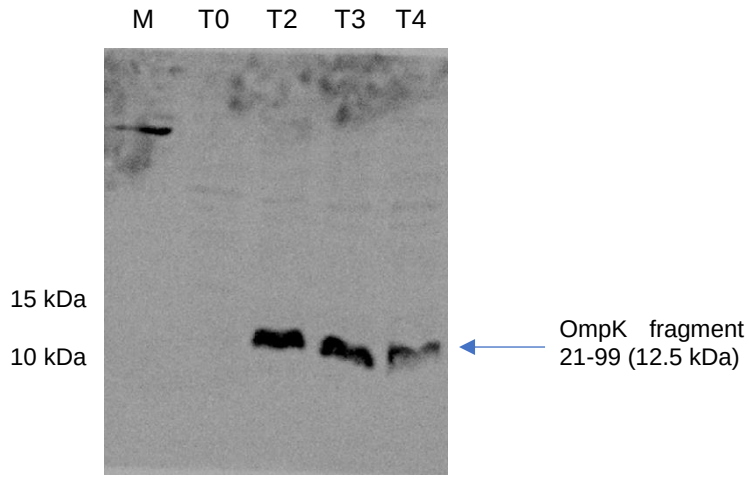


Figure 16 – Western-Blot containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 30 ºC. Lane M: PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher). Lane T0: protein sample taken right before induction; Lanes T2 to T4: protein samples taken during overexpression 2, 3 and 4 hours after induction.

From the analysis of the Western-Blot result, the His-Tagged OmpK fragment 21-99 was successfully expressed in higher amounts comparatively to what was previously obtained for the overexpression at 37 ºC, since bands of the expected size can be observed in the membrane. Consequently, this production condition was repeated and the periplasmic fraction was extracted at the end of the overexpression (T4) to see if the protein was successfully being transported to the periplasmic space, since it possesses a pelB signal peptide.

4.2.2.3. Overexpression at 18 ºC and 25 ºC

Peptide production was also developed at two additional temperatures, with production of the protein whether at 18 ºC or 25 ºC. Protein samples were taken as previously explained with the difference that at 18 ºC only two protein samples were taken from the cell culture: right before induction (T0) and 16 hours after induction (T16), due to the fact that the cells will take a longer time to grow and express the protein. Overexpression at 25 ºC will be stopped 3 hours after induction, with the last protein sample taken at this moment.

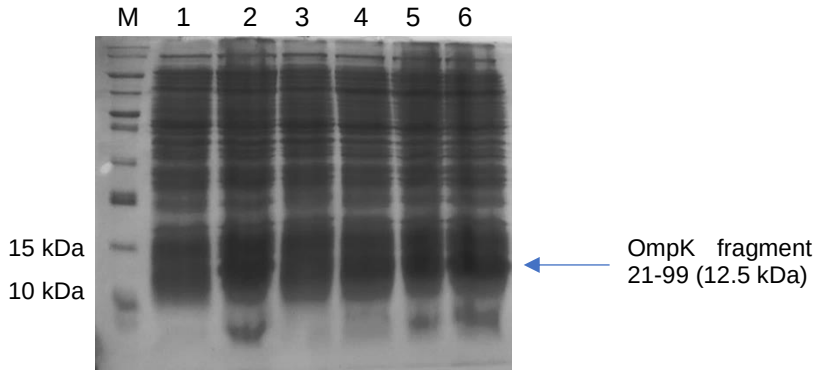


Figure 17 – SDS-PAGE containing protein samples extracted from whole biomass from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 18 ºC and 25 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lane 1: protein sample at T0 of overexpression at 18 ºC; Lane 2: protein sample at T16 of overexpression at 18 ºC; Lane 3: protein sample at T0 of overexpression at 25 ºC; Lanes 4 to 6: protein samples taken during overexpression at 25 ºC, 1, 2 and 3 hours after induction.

From the observation of the figure 17, it is possible to infer that overexpression of the desired peptide occurred, since a band in the expected size is visibly growing in intensity over time. Given that, these two conditions were repeated and the periplasmic fractions were isolated, with the objective of seeing if the peptide would be transported to the periplasmic space after expression.

4.2.2.4. Periplasmic fraction analysis

As mentioned before, the OmpK fragment 21-99 here discussed contains a signal peptide sequence *pelB* in its C-terminal that targets the peptide to the periplasmic space after expression is completed.

The periplasmic fraction was isolated in order to evaluate the presence of the OmpK fragment 21-99 in the periplasm, since its sequence contained a signal peptide *pelB* responsible for the transportation to the periplasmic space. The periplasmic fraction isolation is described in section 3.4.2.4. The periplasmic samples were run in the SDS-PAGE gel and the result is showed in figure 18.

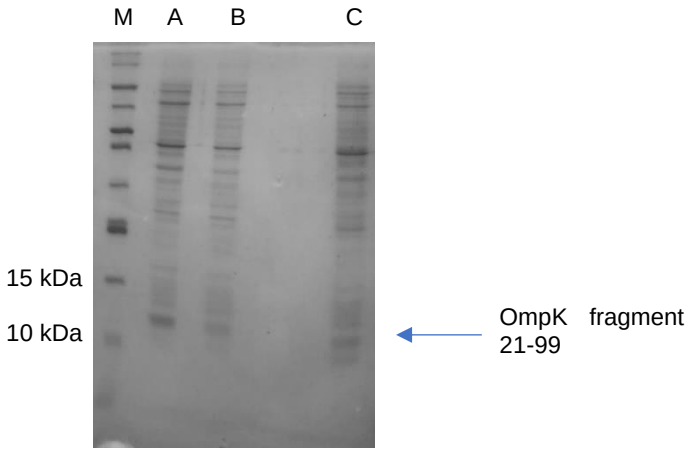


Figure 18 – SDS-PAGE containing periplasmic fraction samples from overexpression in *E. coli* BL21 (DE3) cells with pFCN1 plasmid at 30 ºC, 25 ºC and 18 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lane A: periplasm sample of overexpression at 30 ºC; Lane B: periplasm sample of overexpression at 25 ºC; Lane C: periplasm sample of overexpression at 18 ºC.

The front of the gel was slightly distorted, forming a wave in the area of the lanes A and B, making it seem the bands of the overexpression at 18 ºC are positioned in a lower place comparing to the other samples, when actually the expression profiles are very similar for the different samples. The produced peptide after being transported to the periplasmic space, loses the signal peptide present in the N-terminal and the molecular weight is altered, going from 12.5 kDa to 10.2 kDa. This means that the band corresponding to the desired peptide should be located slightly above the 10 kDa mark of the protein standard, as seems to be the case in figure 18.

To confirm that the bands observed in the electrophoresis gels in figures 17 and 18 correspond to the OmpK fragment being studied, a Western-Blot experiment was conducted in the same conditions as before and figure 19 exhibits the obtained result.

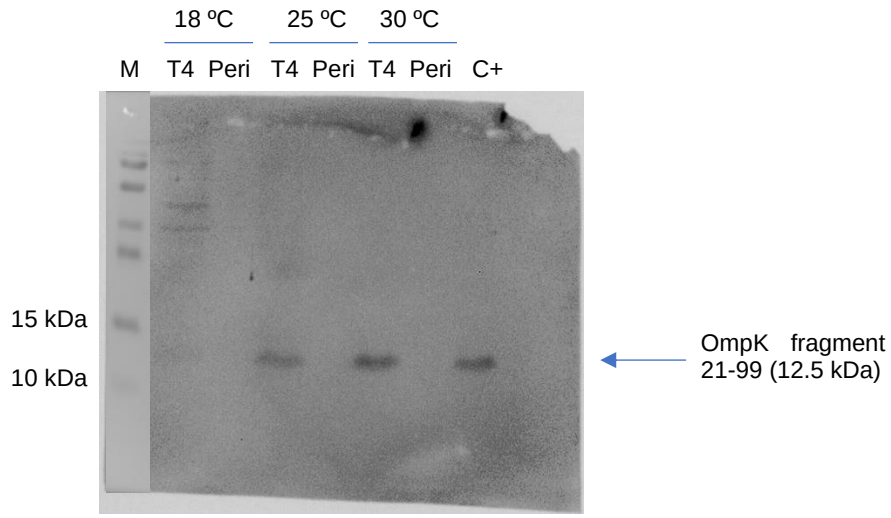


Figure 19 – Western-Blot containing protein samples from overexpression at 18 ºC, 25 ºC and 30 ºC of *E. coli* BL21 (DE3) cells with pFCN1 plasmid. Lane M: PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher); Lane 2: total protein sample T4 overexpression 18 ºC; Lane 3: periplasmic fraction sample overexpression 18 ºC; Lane 4: total protein sample T4 overexpression 25 ºC; Lane 5: periplasmic fraction sample overexpression 25 ºC; Lane 6: total protein sample T4 overexpression 30 ºC; Lane 7: periplasmic fraction sample overexpression 30 ºC; Lane 8: positive control – protein sample T4 from overexpression at 30 ºC.

As expected, the samples corresponding to the total protein extract at 4 hours of overexpression present peptide production. The sample from overexpression at 18 ºC presents a band of weak intensity, and the absence of target protein in the periplasmic fraction. Consequently, this temperature was not selected in the following experiments since the production is not optimal. This conclusion is rather peculiar because normally, the lower the temperature the better the peptide production, since precipitation and formation of inclusion bodies is minimized. Regarding the overexpression at 25 ºC and 30 ºC, the experiments seem to be good to obtain high concentrations of the peptide. However, once the periplasmic fraction is isolated, the peptide fraction diminishes and the signal is lost. This could mean that the peptide might not be present in this fraction, due to an elevated amount of produced peptide, that might be forming aggregates such as inclusion bodies inside the cell cytoplasm (not evaluated). Additionally, it is also possible that the amount of peptide present in the periplasmic fractions is so small that by only taking fractions of 2 mL and concentrating them, might not allow to reach the limit of detection by Western-Blotting analysis. Afterwards, the rest of the periplasmic fraction was concentrated in its totality, mixed with gel loading buffer in the same ratio as before and placed on a western blot. Once again, no band was observed in the expected area, which might indicate that the peptide was not present there at all, meaning that even with the signal peptide associated to the sequence of the fragment, the transport to the periplasm was unsuccessful or that the amount of peptide in the periplasm is too small to be able to be detected. To understand if the band observed in the SDS-PAGE is solely due to the OmpK fragment, an overexpression experiment with *E. coli* BL21 (DE3) cells containing the pET-22b(+) cloning vector was conducted to see if there are other proteins of similar size and that are redirected to the periplasm being highly expressed, that could explain the band observed in the fractions

analyzed. No protein was overexpressed at the same MW of the pFCN1 overexpressed protein. So, it is possible to conclude that the protein is expressed but can not be directed to the periplasm in high amounts and might be present inside the cell as inclusion bodies.

The experiment was conducted as previously described for overexpression at 30 ºC with the pFCN1 plasmid with samples being harvested in similar timepoints. In figure 20 it is possible to observe the protein profile in the cells throughout the overexpression time. Apparently, no protein is being highly expressed with a similar size as the studied OmpK peptide.

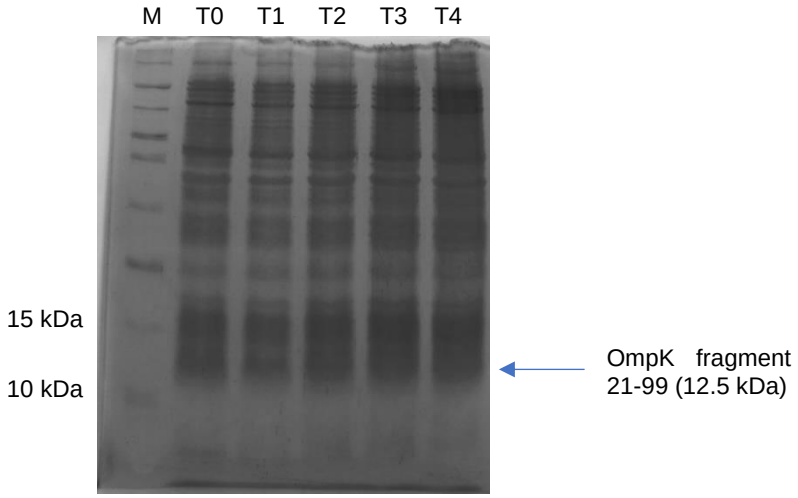


Figure 20 – SDS-PAGE containing whole protein extracts from overexpression in *E. coli* BL21 (DE3) cells with pET-22b(+) plasmid at 30 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 to T4: protein samples taken during overexpression 0, 1, 2, 3 and 4 hours after induction with 0.4 mM IPTG.

Additionally, a Western-Blot experiment was also conducted (figure 21) in order to compare with the samples from the overexpression with the pFCN1 plasmid. This experiment was performed to enable the confirmation that the bands observed in the referred region correspond solely to the OmpK fragment being produced and not to a different protein that is naturally produced. This time, during the Western-Blot procedure, an additional staining step with Ponceau solution was made, right after protein transfer from the gel to the nitrocellulose membrane. This coloration allows an immediate understanding of the success of the transfer before moving on with the experiment, since every protein that is transferred to the membrane appears with a red color, as it is possible to observe in figure 21, upper image. At this stage, it was possible to conclude that the transfer did not work well for the OmpK peptide, since no bands are visible to the naked eye in the expected area. The WB experiment was carried on and the result is displayed in figure 21, in the lower image.

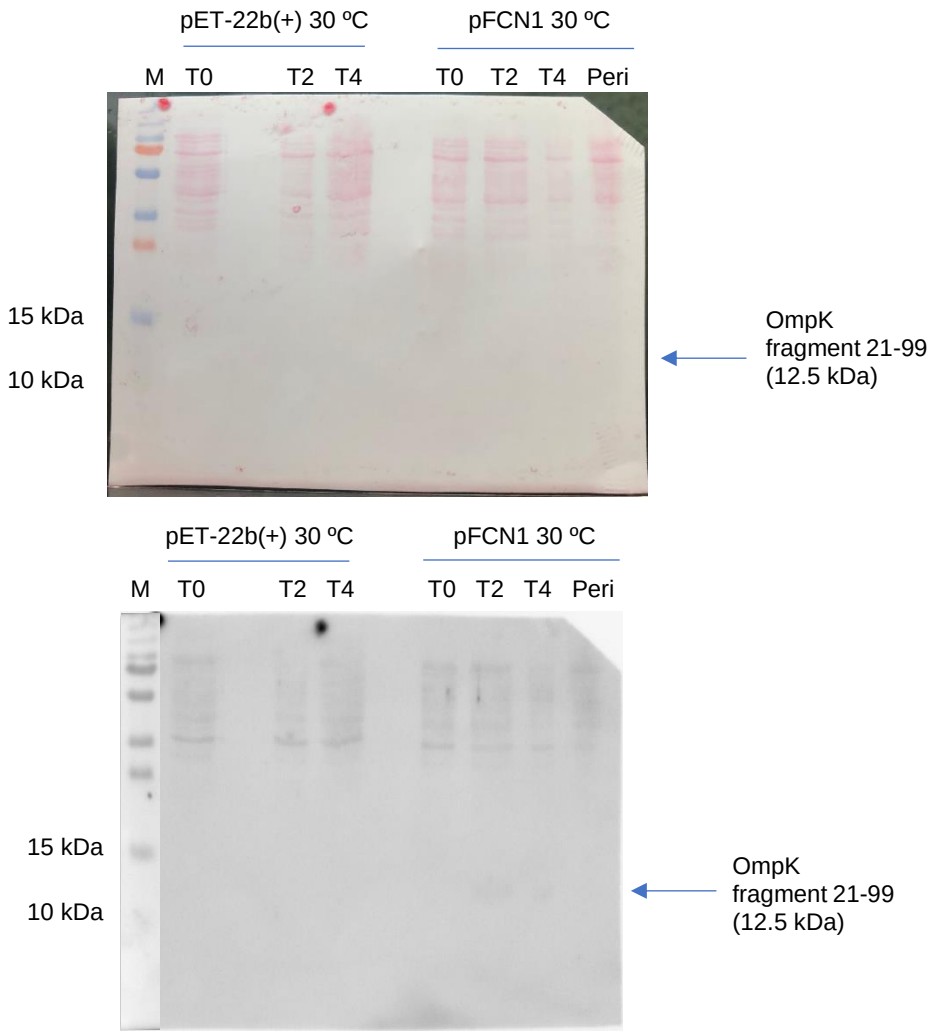


Figure 21 – Western-Blot containing protein extracts from overexpression in *E. coli* BL21 (DE3) cells with pET-22b(+) plasmid at 30 ºC. The upper image shows the Nitrocellulose (NC) membrane after incubation with Ponceau coloration solution. The lower image corresponds to the result of the Western Blot experiment. Lane M: Page Ruler Plus Prestained Protein Ladder (ThermoFisher). Lanes T0 to T4 (pET-22b(+)): whole protein extracts taken during overexpression with the pET-22b(+), at 0, 2 and 4 hours after induction with 0.4 mM IPTG; Lanes T0 to Peri (pFCN1): whole protein extracts taken during overexpression with pFCN1 plasmid at 0, 2 and 4 hours after induction and the periplasmic fraction sample from overexpression with pFCN1 plasmid.

The result is inconclusive since the experiment with Ponceau staining solution did not show a positive transfer of the OmpK peptide to the WB membrane. As mentioned before, this could be associated with troubleshooting problems related with the technique of Western Blotting, which needs to be optimized in the future in order to successfully detect smaller proteins. For instance, it has been reported that low-molecular peptides are more difficult to detect using the conventional Western blotting method since they are readily detached from the blotted membranes, making PVDF membranes more optimal to overcome those detachment problems (Tomisawa et al., 2013). Different WB protocols, such as semi-dry transfer (with lower transfer times), or vaccum-assisted method (shorter immunodetection time), might work better for small peptides (Tomisawa et al., 2013). Furthermore, the presence of a high

quantity of background in the membrane, as it is recurrent throughout the experiments, can be an indicator of a problem related with various factors, such as excessive antibody concentration, long exposure times, incomplete blocking or even improper washing (Signore & Hodge, 2017). Hence, the Western Blot technique should be repeated and optimized.

Figure 22 presents the SDS-PAGE gel containing the samples from the periplasmic fraction isolation. Although the resolution is not the best, the samples from the overexpression with the cloning vector pET-22b(+) confirm the absence of a protein with similar size as the OmpK peptide. Additionally, comparing the periplasmic fractions from the different experiments, only the overexpression with pFCN1 presents a band in the MW expected region. As expected, this strategy allowed to confirm that the peptide produced in the previous experiments corresponds in fact to the OmpK, and its absence from the periplasmic fraction must be due to a problem in the isolation step, a failure of secretion to the periplasm or a problem at the detection step.

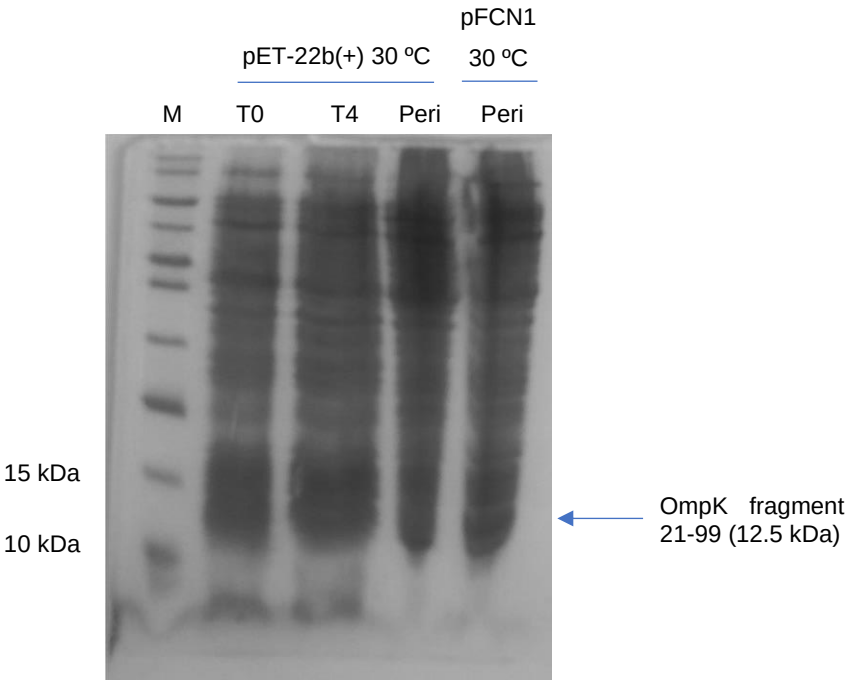


Figure 22 – SDS-PAGE containing protein extracts from overexpression in *E. coli* BL21 (DE3) cells with pET-22b(+) plasmid at 30 ºC. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 and T4: protein extracts taken during overexpression with pET-22b(+), 0 and 4 hours after induction with 0.4 mM IPTG. Lane Peri (pET-22b(+)): periplasmic fraction sample from overexpression with pET-22b(+) plasmid. Lane Peri (pFCN1): periplasmic fraction sample from overexpression with pFCN1 plasmid.

4.2.2.5. Overexpression at 30 ºC in *E. coli* BL21 (DE3) pSAS39 cells with the pFCN1 plasmid

Overexpression of the peptide was also developed in *E. coli* BL21 (DE3) cells already containing the pSAS39 plasmid that enables the production of an antisense molecule, reportedly diminishing the production of the Lpp molecule from the cell outer membrane, causing an effect of cell permeabilization.

This effect will allow an easier transport of certain substances through the cell membrane to the extracellular medium, which would consequently allow an easier recovery of the produced OmpK fragment. Since the overexpression at 30 ºC showed really good results, this condition was the one chosen to undertake this new strategy.

Figure 23 shows the SDS-PAGE electrophoresis gel containing the whole protein sample extracts from *E. coli* taken during the experiment. For terms of comparison, an additional cell culture (control) was developed with the difference that the production of antisense was not induced in order to see if this molecule was actually having an effect on the presence of the produced peptide in the extracellular medium. The inducer arabinose 2 %, was added 2 hours after inducing the production of the peptide.

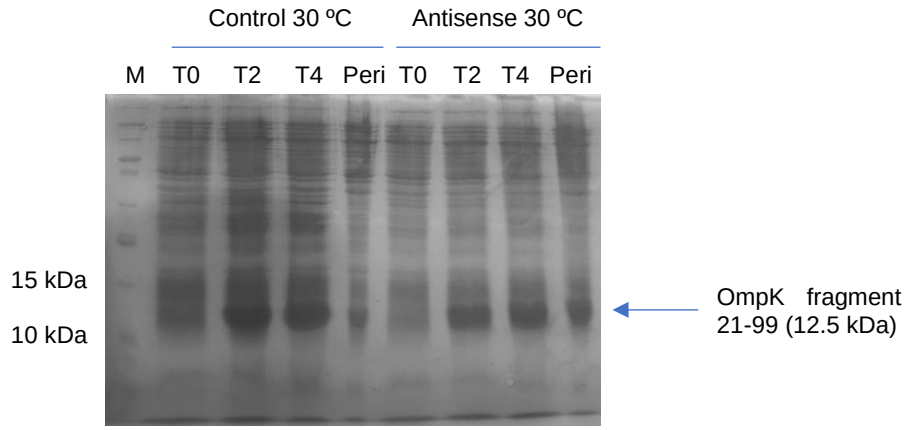


Figure 23 – SDS-PAGE containing protein extracts from overexpression at 30 ºC of *E. coli* BL21 (DE3) pSAS39 cells + pFCN1. Lane M: Page ruler Unstained Broad Range (ThermoFisher); Lanes T0 to T4 (control): protein samples at T0, T2 and T4 of overexpression control at 30 ºC; Lane Peri (Control): periplasmic fraction of overexpression control at 30 ºC; Lane T0 to T4 (Antisense): protein samples at T0, T2 and T4 of overexpression with antisense at 30 ºC; Lane Peri Antisense: periplasmic fraction of overexpression with antisense at 30 ºC.

Peptide production was observed in both experiments, with and without induction of antisense production. In both cases, a high intensity band in the expected size area can be seen, and its intensity is increasing from the sample T2 to the T4 (figure 23). Similarly, the samples corresponding to the periplasmic fraction appear to contain the peptide, which could mean that the peptide is being redirected to the periplasmic space, as an effect of the signal peptide present in the N-terminal of the OmpK fragment 21-99, which is then cleaved once the peptide reaches the periplasmic space.

To make sure that the produced protein corresponds to the desired peptide, a Western-Blot experiment was conducted (figure 24) in which only proteins with a His-Tag in its extremity would be recognized, as is the case of the peptide being produced in this work.

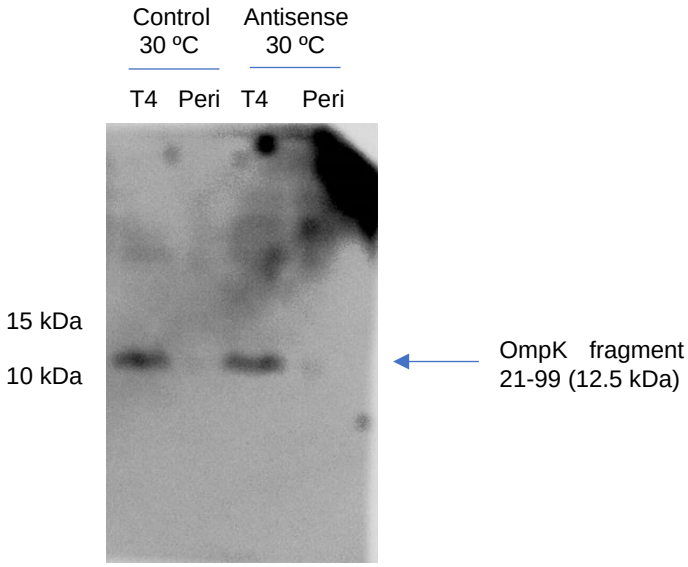


Figure 24 – Western-Blot containing protein extracts from overexpression of *E. coli* BL21 (DE3) cells with pSAS39 + pFCN1 at 30 ºC, with and without antisense induction. Lane T4 (Control): protein sample T4 overexpression control 30 ºC; Lane Peri (Control): periplasmic fraction sample overexpression control 30 ºC; Lane T4 (antisense): protein sample T4 overexpression with antisense 30 ºC; Lane Peri (Antisense): periplasmic fraction sample overexpression with antisense 30 ºC.

As it is possible to observe in figure 24, in both overexpression experiments the desired peptide was successfully produced since a band of the expected size is observed in the samples corresponded to the whole protein extracts after 4 hours of protein induction. On the other hand, the periplasmic fraction samples present a band in the same size MW but of very low intensity. This could mean that, once more, although the protein is being produced by the cell, it might not be transported to the periplasmic space in high amounts or that the process of periplasmic fraction isolation results in a loss of the produced peptide, making it unable to be observed in the Western-Blot.

The extracellular medium was isolated as well in the end of the peptide overexpression with the objective of analyzing the antisense effect on the outer cell membrane. A 2 mL sample fraction was concentrated, mixed with gel loading buffer and placed on a Western-Blot. Once again, no band was observed, which might mean that, similarly to the periplasmic fraction, the amount of peptide in the extracellular medium might not be enough to result in a signal in the Western-Blot.

Whether the antisense has an effect on the cell is inconclusive since the peptide is not being successfully transported to the periplasm in high amounts, as it would be expected. If the peptide was present in the periplasm, the effect of the antisense molecule could be evaluated by checking its presence in the extracellular medium, and eventually understand if the antisense was successfully causing a permeabilization effect on the outer cell membrane. The entire extracellular medium fraction was concentrated and the sample used for a Western-Blot which demonstrated that the protein was absent from the extracellular medium, possibly meaning that the antisense molecule was not enough to cause a permeabilization effect on the cell membrane. The exact cause for this still has to be better

studied but can be due to various factors, such as loss of the plasmid containing the antisense sequence or be related with the conditions, such as time or concentration of the antisense induction, that should be more well studied. Additionally, it can also be related with a trouble in the Western Blot technique that should be better well studied in order to understand if the exact conditions and materials typically used for protein detection can also be applied to smaller peptides.

5. Conclusion and Future Perspectives

Construction of the plasmid with the lpp antisense was successfully obtained, which allowed the evaluation of the effect this sequence was causing on *E. coli* BL21 (DE3) cells and more specifically, on the Lpp Braun lipoprotein from the cells' outer membrane. Monitorization of growth was achieved by measuring the OD_{600nm} over time, showing slight differences in the specific growth rates of *E. coli* BL21 (DE3) wild type with and without induction, and depending on the presence of the pMLBAD vector or the pSAS39 plasmid. Generally, the wild-type culture without induction showed a higher growth than the other cultures, indicating that the arabinose added to induce the production of the antisense is also consumed by the production cells as part of its metabolism. This hypothesis is supported by the results obtained in the arabinose analysis of the culture media by HPLC, that demonstrated a slight decrease in the arabinose concentration in the medium. As the essays were only conducted once for each condition, the procedures should be repeated to guarantee this behaviour. Additional susceptibility studies should be conducted in order to properly evaluate the antisense effect on the membrane.

Furthermore, the values of lpp mRNA expression were determined by RT-PCR. Samples collected from the culture media with production of the pMLBAD or pSAS39, after 2 h of the first induction and 1.5 h after the second induction were used for the determination of the lpp mRNA levels. A clear reduction of the lpp transcript was observed since its amount is reduced nearly a third in only 2 hours. However, after 1.5 h of the second induction, the mRNA levels return to the normality. As explained, this could be related with the fact that the amount of antisense produced per cell is variable, with cells not producing anything at all (Doherty et al., 1993; Dong et al., 1995). Since the antisense inhibits the production of a protein involved in the outer-membrane structure, it is possible that the subpopulation of cells with high synthesis of the antisense disappears and the effect of the antisense is lost, hence explaining the stabilization of the lpp mRNA values at 1.5 h after the second induction. The experiment should be repeated in order to confirm the antisense effect and different conditions of growth, with induction at different moments or with different inducer concentrations, should be performed to get a better perspective on the actual effect posed by the antisense.

Overproduction of the OmpK 21-99 fragment was carried out at several different temperatures (37 ºC, 30 ºC, 25 ºC and 18 ºC), in order to evaluate which condition would be the best to produce large quantities of the peptide. After SDS-PAGE and Western Blot analysis, the temperature 30 ºC was determined to be the best in producing the target peptide, although the peptide was not observed in the periplasm (as it was expected since it contained the *pelB* signal sequence). This temperature was chosen to carry out the same experiment in *E. coli* BL21 (DE3) + pSAS39 plasmid, with induction of the antisense production. Peptide expression was confirmed but analysis of the periplasm and cytoplasm did not show the presence of the peptide in these fractions, indicating periplasm transport was not successful or that the amount of peptide that did go to the periplasm was not enough to be detected by Western Blot. This procedure demonstrated certain issues in the detection step, with long detection times, weak signals in the membrane and a lot of unspecific bonds, which might indicate that the peptide is not being successfully transferred into the membrane. In the future, this technique will have to be

optimized for transfer of small peptides, possibly by using a PVDF membrane or by changing certain conditions in the procedure, such as lowering incubation times or temperature (Tomisawa et al., 2013).

6. Bibliography

Ahmed, N., & Thompson, S. (2019). The blue dimensions of aquaculture: A global synthesis. *Science of the Total Environment*, *652*, 851–861. https://doi.org/10.1016/j.scitotenv.2018.10.163

Albuquerque Costa, R., Araújo, R. L., Souza, O. V., & Vieira, R. H. S. D. F. (2015). Antibiotic-resistant vibrios in farmed shrimp. *BioMed Research International*, *2015*(505914). https://doi.org/10.1155/2015/505914

Asmar, A. T., & Collet, J. F. (2018). Lpp, the Braun lipoprotein, turns 50—major achievements and remaining issues. *FEMS Microbiology Letters*, *365*(18), 1–8. https://doi.org/10.1093/femsle/fny199

Bennett, C. F. (2019). Therapeutic Antisense Oligonucleotides Are Coming of Age. *Annual Review of Medicine*, *70*, 207–321. https://doi.org/10.1146/annurev-med-041217-010829

Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H., & Kompala, D. S. (1989). Plasmid-encoded protein: the principal factor in the “metabolic burden” associated with recombinant bacteria. Biotechnology Bioengineering, 1990. *Biotechnol Bioeng*, *102*(5), 1284–1297; discussion 1283. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19215036

Birnbaum, S., & Bailey, J. E. (1991). Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant *Escherichia coli*. *Biotechnology and Bioengineering*, *37*(8), 736–745. https://doi.org/10.1002/bit.260370808

Blommel, P. G., & Fox, B. G. (2007). A combined approach to improving large-scale production of tobacco etch virus protease. *Protein Expression and Purification*, *55*(1), 53–68. https://doi.org/10.1016/j.pep.2007.04.013

Bucher, M. H., Evdokimov, A. G., & Waugh, D. S. (2002). Differential effects of short affinity tags on the crystallization of *Pyrococcus furiosus* maltodextrin-binding protein. *Acta Crystallographica Section D: Biological Crystallography*, *58*(3), 392–397. https://doi.org/10.1107/S0907444901021187

Camps, M. (2010). Modulation of ColE1-like plasmid replication for recombinant gene expression. *Recent Patents on DNA and Gene Sequences*, *4*(1), 58–73. https://doi.org/10.2174/187221510790410822

Cascales, E., Bernadac, A., Gavioli, M., Lazzaroni, J. C., & Lloubes, R. (2002). Pal lipoprotein of *Escherichia coli* plays a major role in outer membrane integrity. *Journal of Bacteriology*, *184*(3), 754–759. https://doi.org/10.1128/JB.184.3.754-759.2002

Chen, G., Patten, C. L., & Schellhorn, H. E. (2003). Controlled Expression of an *rpoS* Antisense RNA Can Inhibit RpoS Function in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, *47*(11), 3485–3493. https://doi.org/10.1128/AAC.47.11.3485-3493.2003

Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: Structural and mechanistic insights. *Nature Reviews Microbiology*, *13*(6), 343–359. https://doi.org/10.1038/nrmicro3456

Davis, G. D., Elisee, C., Mewham, D. M., & Harrison, R. G. (1999). New fusion protein systems designed to give soluble expression in *Escherichia coli*. *Biotechnology and Bioengineering*, *65*(4), 382–388. https://doi.org/10.1002/(SICI)1097-0290(19991120)65:4<382::AID-BIT2>3.0.CO;2-I

de Boer, H. A., Comstock, L. J., & Vasser, M. (1983). The tac promoter: a functional hybrid derived from the trp and lac promoters. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(1), 21–25. https://doi.org/10.1073/pnas.80.1.21

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., & Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology*, *25*(10), 472–479. https://doi.org/10.1016/j.tibtech.2007.08.001

Demain, A. L., & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*, *27*(3), 297–306. https://doi.org/10.1016/j.biotechadv.2009.01.008

Denman, S. E., & McSweeney, C. S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology*, *58*(3), 572–582. https://doi.org/10.1111/j.1574-6941.2006.00190.x

Dias, N., & Stein, C. A. (2002). Antisense oligonucleotides: Basic concepts and mechanisms. *Molecular Cancer Therapeutics*, *1*(5), 347–355.

Doherty, A. J., Connolly, B. A., & Worrall, A. F. (1993). Overproduction of the toxic protein, bovine pancreatic DNaseI, in *Escherichia coli* using a tightly controlled T7-promoter-based vector. *Gene*, *136*(1–2), 337–340. https://doi.org/10.1016/0378-1119(93)90491-K

Dong, H., Nilsson, L., & Kurland, C. G. (1995). Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *Journal of Bacteriology*, *177*(6), 1497–1504. https://doi.org/10.1128/jb.177.6.1497-1504.1995

LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. & McCoy, J. M. (1993). A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the *E. coli* Cytoplasm. *Nature Biotechnology*, *11*, 187–193.

Francis, M. J. (2018). Recent Advances in Vaccine Technologies. *Veterinary Clinics of North America: Small Animal Practice*, *48*(2), 231–241. https://doi.org/10.1016/j.cvsm.2017.10.002

Georgiou, G., & Segatori, L. (2005). Preparative expression of secreted proteins in bacteria: Status report and future prospects. *Current Opinion in Biotechnology*, *16*(5), 538–545. https://doi.org/10.1016/j.copbio.2005.07.008

Gottesman, S. (1996). Proteases and their targets in *Escherichia coli*. *Annual Review of Genetics*, *30*, 465–506. https://doi.org/10.1146/annurev.genet.30.1.465

Grodberg, J., & Dunn, J. J. (1988). *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *Journal of Bacteriology*, *170*(3), 1245–1253. https://doi.org/10.1128/jb.170.3.1245-1253.1988

Guzman, L. M., Weiss, D. S., & Beckwith, J. (1995). Tight Regulation, Modulation, and High-Level Expression by Vectors Containg the Arabinose pBAD Promoter. *Journal of Bacteriology*, *177*(14), 4121–4130. https://doi.org/10.1128/jb.179.16.5094-5103.1997

Hammarström, M., Hellgren, N., van den Berg, S., Berglund, H., & Härd, T. (2009). Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*. *Protein Science*, *11*(2), 313–321. https://doi.org/10.1110/ps.22102

Hamod, M. A., Nithin, M. S., Shukur, Y. N., Karunasagar, I., & Karunasagar, I. (2012). Outer membrane protein K as a subunit vaccine against *V. anguillarum*. *Aquaculture*, *354–355*, 107–110. https://doi.org/10.1016/j.aquaculture.2012.03.033

Hegarty, J. P., & Stewart, D. B. (2018). Advances in therapeutic bacterial antisense biotechnology. *Applied Microbiology and Biotechnology*, *102*(3), 1055–1065. https://doi.org/10.1007/s00253-017-8671-0

Hong, H., Patel, D. R., Tamm, L. K., & Van Den Berg, B. (2006). The outer membrane protein OmpW forms an eight-stranded β-barrel with a hydrophobic channel. *Journal of Biological Chemistry*, *281*(11), 7568–7577. https://doi.org/10.1074/jbc.M512365200

Huehn, S., Eichhorn, C., Urmersbach, S., Breidenbach, J., Bechlars, S., Bier, N., Alter, T., Bartelt, E., Frank, C., Oberheitmann, B., Gunzer, F., Brennholt, N., Boer, S., Appel, B., Dieckmann, R., & Strauch, E. (2014). Pathogenic vibrios in environmental, seafood and clinical sources in Germany. *International Journal of Medical Microbiology*, *304*(7), 843–850. https://doi.org/10.1016/j.ijmm.2014.07.010

Hwang, P. M., Pan, J. S., & Sykes, B. D. (2014). Targeted expression, purification, and cleavage of fusion proteins from inclusion bodies in *Escherichia coli*. *FEBS Letters*, *588*(2), 247–252. https://doi.org/10.1016/j.febslet.2013.09.028

Inoue, T., Matsuzaki, S., & Tanaka, S. (1995a). A 26-kDa outer membrane protein, OmpK, common to *Vibrio* species is the receptor for a broad-host-range vibriophage, KVP40. *FEMS Microbiology Letters*, *125*(1), 101–105. https://doi.org/10.1016/0378-1097(94)00480-F

Inoue, T., Matsuzaki, S., & Tanaka, S. (1995b). Cloning and sequence analysis of *Vibrio parahaemolyticus* ompK gene encoding a 26-kDa outer membrane protein, OmpK, that serves as receptor for a broad-host-range vibriophage, KVP40. *FEMS Microbiology Letters*, *134*(2–3), 245–249. https://doi.org/10.1016/0378-1097(95)00414-9

Jenny, R. J., Mann, K. G., & Lundblad, R. L. (2003). A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. *Protein Expression and Purification*, *31*(1), 1–11. https://doi.org/10.1016/S1046-5928(03)00168-2

Ji, Q., Wang, S., Ma, J., & Liu, Q. (2020). A review: Progress in the development of fish *Vibrio* spp. vaccines. *Immunology Letters*, *226*(April), 46–54. https://doi.org/10.1016/j.imlet.2020.07.002

Juan, C., Torrens, G., Barceló, M., & Oliver, A. (2018). Interplay between Peptidoglycan Biology and Virulence in Gram-Negative Pathogens. *Microbiology and Molecular Biology Reviews*, *82*(4). https://doi.org/10.1128/MMBR.00033-18

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology*, *6*(5), 494–500. https://doi.org/10.1016/0958-1669(95)80082-4

Kapust, R. B., & Waugh, D. S. (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused . *Protein Science*, *8*(8), 1668–1674. https://doi.org/10.1110/ps.8.8.1668

Khan, F., Legler, P. M., Mease, R. M., Duncan, E. H., Bergmann-Leitner, E. S., & Angov, E. (2012). Histidine affinity tags affect MSP1 42 structural stability and immunodominance in mice. *Biotechnology Journal*, *7*(1), 133–147. https://doi.org/10.1002/biot.201100331

Khlebnikov, A., Risa, Skaug, T., Carrier, T. A., & Keasling, J. D. (2000). Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. *Journal of Bacteriology*, *182*(24), 7029–7034. https://doi.org/10.1128/JB.182.24.7029-7034.2000

Kleiner-Grote, G. R. M., Risse, J. M., & Friehs, K. (2018). Secretion of recombinant proteins from *E. coli*. *Engineering in Life Sciences*, *18*(8), 532–550. https://doi.org/10.1002/elsc.201700200

Klose, J., Wendt, N., Kubald, S., Krause, E., Fechner, K., Beyermann, M., Bienert, M., Rudolph, R. & Rothemund, S. (2004). Hexa-histidin tag position influences disulfide structure but not binding behavior of in vitro folded N-terminal domain of rat corticotropin-releasing factor receptor type 2a. *Protein Science*, *13*(9), 2470–2475. https://doi.org/10.1110/ps.04835904

Korpimäki, T., Kurittu, J., & Karp, M. (2003). Surprisingly fast disappearance of β-lactam selection pressure in cultivation as detected with novel biosensing approaches. *Journal of Microbiological Methods*, *53*(1), 37–42. https://doi.org/10.1016/S0167-7012(02)00213-0

Lanzer, M., & Bujard, H. (1988). Promoters largely determine the efficiency of repressor action. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(23), 8973–8977. https://doi.org/10.1073/pnas.85.23.8973

Le Roux, F., & Blokesch, M. (2018). Eco-evolutionary Dynamics Linked to Horizontal Gene Transfer in Vibrios. *Annual Review of Microbiology*, *72*, 89–110. https://doi.org/10.1146/annurev-micro-090817-062148

Lefebre, M. D., & Valvano, M. A. (2002). Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. *Applied and Environmental Microbiology*, *68*(12), 5956–5964. https://doi.org/10.1128/AEM.68.12.5956-5964.2002

Li, N., Yang, Z., Bai, J., Fu, X., Liu, L., Shi, C., & Wu, S. (2010). A shared antigen among *Vibrio* species: Outer membrane protein-OmpK as a versatile Vibriosis vaccine candidate in Orange-spotted grouper (*Epinephelus coioides*). *Fish and Shellfish Immunology*, *28*(5–6), 952–956. https://doi.org/10.1016/j.fsi.2010.02.010

Li, Y. D., Ren, H. L., Lu, S. Y., Zhou, Y., Han, X., Gong, B. B., Zhang, Y. Y. & Liu, Z. S. (2010). Cloning, expression, and genus-specificity analysis of 28-kDa ompK from *Vibrio alginolyticus*. *Journal of Food Science*, *75*(4), 198–203. https://doi.org/10.1111/j.1750-3841.2010.01565.x

Liljeqvist, S., & Ståhl, S. (1999). Production of recombinant subunit vaccines: Protein immunogens, live delivery systems and nucleic acid vaccines. *Journal of Biotechnology*, *73*(1), 1–33. https://doi.org/10.1016/S0168-1656(99)00107-8

Lopes, J. F. (2017). *Studies with Vibrio alginolyticus OmpK towards the development of a vaccine for Solea senegalensis*.

Maniatis, T., Fritsch E. F. and Sambrook, J. K. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Mao, Z., Yu, L., You, Z., Wei, Y., & Liu, Y. (2007). Cloning, expression and immunogenicty analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. *Fish and Shellfish Immunology*, *23*(3), 567–575. https://doi.org/10.1016/j.fsi.2007.01.004

Markides, S. C. (1996). Strategies for Achieving High-Level Expression of Genes in *Escherichia Coli*. *Microbiological Reviews*, *60*(3), 512–538.

Mergulhão, F. J. M., Summers, D. K., & Monteiro, G. A. (2005). Recombinant protein secretion in *Escherichia coli*. *Biotechnology Advances*, *23*(3), 177–202. https://doi.org/10.1016/j.biotechadv.2004.11.003

Munn, C. B. (2015). The Role of Vibrios in Diseases of Corals. *Microbiology Spectrum*, *3*(4), 1–12. https://doi.org/10.1128/microbiolspec.ve-0006-2014

Ni, Y., & Chen, R. (2009). Extracellular recombinant protein production from *Escherichia coli*. *Biotechnology Letters*, *31*(11), 1661–1670. https://doi.org/10.1007/s10529-009-0077-3

Ni, Y., Reye, J., & Chen, R. R. (2007). Lpp deletion as a permeabilization method. *Biotechnology and Bioengineering*, *97*(6), 1347–1356. https://doi.org/10.1002/bit21375

Ningqiu, L., Junjie, B., Shuqin, W., Xiaozhe, F., Haihua, L., Xing, Y., & Cunbin, S. (2008). An outer membrane protein, OmpK, is an effective vaccine candidate for *Vibrio harveyi* in Orange-spotted grouper (*Epinephelus coioides*). *Fish and Shellfish Immunology*, *25*(6), 829–833. https://doi.org/10.1016/j.fsi.2008.09.007

Petsch, D., & Anspach, F. B. (2000). Endotoxin removal from protein solutions. *Journal of Biotechnology*, *76*(2–3), 97–119. https://doi.org/10.1016/S0168-1656(99)00185-6

Peubez, I., Chaudet, N., Mignon, C., Hild, G., Husson, S., Courtois, V., De Luca, K., Speck, D. & Sodoyer, R. (2010). Antibiotic-free selection in *E. coli*: New considerations for optimal design and improved production. *Microbial Cell Factories*, *9*, 1–10. https://doi.org/10.1186/1475-2859-9-65

Pope, B., & Kent, H. M. (1996). High efficiency 5 min transformation of *Escherichia coli*. *Nucleic Acids Research*, *24*(3), 536–537. https://doi.org/10.1093/nar/24.3.536

Qian, R. H., Xiao, Z. H., Zhang, C. W., Chu, W. Y., Wang, L. S., Zhou, H. H., Wei, Y. W. & Yu, L. (2008). A conserved outer membrane protein as an effective vaccine candidate from *Vibrio alginolyticus*. *Aquaculture*, *278*(1–4), 5–9. https://doi.org/10.1016/j.aquaculture.2008.03.010

Rasmussen, L. C. V., Sperling-Petersen, H. U., & Mortensen, K. K. (2007). Hitting bacteria at the heart of the central dogma: Sequence-specific inhibition. *Microbial Cell Factories*, *6*(24), 1–26. https://doi.org/10.1186/1475-2859-6-24

Reisenbauer, A. (2018). *Development of an Eschrichia coli strain to express bacterial antigenic peptide and co-express Braun's Lipoprotein Antisense oligonucleotide*.

Riguero, V., Clifford, R., Dawley, M., Dickson, M., Gastfriend, B., Thompson, C., Wang, S. C. & O'Connor, E. (2020). Immobilized metal affinity chromatography optimization for poly-histidine tagged proteins. *Journal of Chromatography A*, *1629*, 461505. https://doi.org/10.1016/j.chroma.2020.461505

Roberts, M. C. (1996). Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Reviews*, *19*(1), 1–24. https://doi.org/10.1016/0168-6445(96)00021-6

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*, *5*(APR), 1–17. https://doi.org/10.3389/fmicb.2014.00172

Schleif, R. (2010). AraC protein, regulation of the L-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action. *FEMS Microbiology Reviews*, *34*, 779–796. https://doi.org/10.1111/j.1574-6976.2010.00226.x

Sha, J., Agar, S. L., Baze, W. B., Olano, J. P., Fadl, A. A., Erova, T. E., Wang, S., Foltz, S. M., Suarez, G., Motin, V. L., Chauhan, S., Klimpel, G. R., Peterson, J. W. & Chopra, A. K. (2008). Braun lipoprotein (Lpp) contributes to virulence of *yersiniae*: Potential role of Lpp in inducing bubonic and pneumonic plague. *Infection and Immunity*, *76*(4), 1390–1409. https://doi.org/10.1128/IAI.01529-07

Shin, H. D., & Chen, R. R. (2008). Extracellular recombinant protein production from an *Escherichia coli* lpp deletion mutant. *Biotechnology and Bioengineering*, *101*(6), 1288–1296. https://doi.org/10.1002/bit.22013

Shokri, A., Sandén, A. M., & Larsson, G. (2003). Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. *Applied Microbiology and Biotechnology*, *60*(6), 654–664. https://doi.org/10.1007/s00253-002-1156-8

Signore, M., & Hodge, A. (2017). Antibody validation by Western blotting. *Methods in Molecular Biology*, *1606*, 51–70. https://doi.org/10.1007/978-1-60327-216-2_10

Silvaraj, S., Yasin, I. S. M., Karim, M. M. A., & Saad, M. Z. (2020). Elucidating the efficacy of vaccination against vibriosis in lates calcarifer using two recombinant protein vaccines containing the outer membrane protein k (R-ompk) of *vibrio alginolyticus* and the dna chaperone j (r-dnaj) of *vibrio harveyi*. *Vaccines*, *8*(4), 1–19. https://doi.org/10.3390/vaccines8040660

Singh, S. M., & Panda, A. K. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, *99*(4), 303–310. https://doi.org/10.1263/jbb.99.303

Smith, D. B., & Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, *67*(1), 31–40. https://doi.org/10.1016/0378-1119(88)90005-4

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Review of Vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Stein, A. C. A., & Cheng, Y. (1993). Antisense Oligonucleotides as Therapeutic Agents-Is the Bullet Really Magical ? *Science*, *261*(5124), 1004–1012. https://doi.org/10.1126/science.8351515

Terpe, K. (2003). Overview of tag protein fusions: From molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, *60*(5), 523–533. https://doi.org/10.1007/s00253-002-1158-6

Terpe, Kay. (2006). Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, *72*(2), 211–222. https://doi.org/10.1007/s00253-006-0465-8

Thompson, F L, Gevers, D., Thompson, C. C., Dawyndt, P., Naser, S., Hoste, B., Munn, C. B. & Swings, J. (2005). Phylogeny and Molecular Identification of Vibrios on the Basis of Multilocus Sequence Analysis. *Applied and Environmental Microbiology*, *71*(9), 5107–5115. https://doi.org/10.1128/AEM.71.9.5107

Thompson, Fabiano L., Iida, T., & Swings, J. (2004). Biodiversity of Vibrios. *Microbiology and Molecular Biology Reviews*, *68*(3), 403–431. https://doi.org/10.1128/mmbr.68.3.403-431.2004

Tomisawa, S., Abe, C., Kamiya, M., Kikukawa, T., Demura, M., Kawano, K., & Aizawa, T. (2013). A new approach to detect small peptides clearly and sensitively by Western blotting using a vacuum-assisted detection method. *Biophysics (Japan)*, *9*, 79–83. https://doi.org/10.2142/biophysics.9.79

Tůma, S., Izaguirre, J. K., Bondar, M., Marques, M. M., Fernandes, P., da Fonseca, M. M. R., & Cesário, M. T. (2020). Upgrading end-of-line residues of the red seaweed *Gelidium sesquipedale* to polyhydroxyalkanoates using *Halomonas boliviensis*. *Biotechnology Reports*, *27*. https://doi.org/10.1016/j.btre.2020.e00491

Uhlich, G. A., Gunther IV, N. W., Bayles, D. O., & Mosier, D. A. (2009). The CsgA and lpp proteins of an *escherichia coli* O157:H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. *Infection and Immunity*, *77*(4), 1543–1552. https://doi.org/10.1128/IAI.00949-08

Wang, H., Li, X., Ma, Y., & Song, J. (2014). Process optimization of high-level extracellular production of alkaline pectate lyase in recombinant *Escherichia coli* BL21 (DE3). *Biochemical Engineering Journal*, *93*, 38–46. https://doi.org/10.1016/j.bej.2014.08.020

Xu, Z., & Kulp, D. W. (2019). Protein engineering and particulate display of B-cell epitopes to facilitate development of novel vaccines. *Current Opinion in Immunology*, *59*, 49–56. https://doi.org/10.1016/j.coi.2019.03.003

Yang, H., Lu, X., Hu, J., Chen, Y., Shen, W., & Liu, L. (2018). Boosting secretion of extracellular protein by *Escherichia coli* via cell wall perturbation. *Applied and Environmental Microbiology*, *84*(20), 1–14. https://doi.org/10.1128/AEM.01382-18

Yuan, J. S., Reed, A., Chen, F., & Stewart, C. N. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics*, *7*, 1–12. https://doi.org/10.1186/1471-2105-7-85

Zhang, C., Yu, L., & Qian, R. (2007). Characterization of OmpK, GAPDH and their fusion OmpK-GAPDH derived from *Vibrio harveyi* outer membrane proteins: Their immunoprotective ability against vibriosis in large yellow croaker (*Pseudosciaena crocea*). *Journal of Applied Microbiology*, *103*(5), 1587–1599. https://doi.org/10.1111/j.1365-2672.2007.03386.x

Zou, C., Duan, X., & Wu, J. (2014). Enhanced extracellular production of recombinant *Bacillus deramificans* pullulanase in *Escherichia coli* through induction mode optimization and a glycine feeding strategy. *Bioresource Technology*, *172*, 174–179. https://doi.org/10.1016/j.biortech.2014.09.035

7. Annexes

Annex 1 – An arabinose calibration curve was constructed in HPLC to obtain a calibration rate to be able to calculate concentration of arabinose in the culture media.

Table A1 – The left table containing values of concentration of arabinose solutions prepared in distilled water for creation of the calibration curve. AU is the arbitrary units in which peaks area are expressed. The right table contains the values obtained for the retention time (rt) of arabinose, m is the slope of the calibration curve and b is the intersection with the y axis.

[Arabinose] (g/L)	A (AU)
0	0
5	438047
10	804736
15	1400860
20	1191517
40	3307700
80	12399157
120	18987452
160	23040454
200	36177432

rt	15.3 min
m	5.60 x 10^{-6} g/(L.peak area)
b	1.14 x 10^{+01} g/L

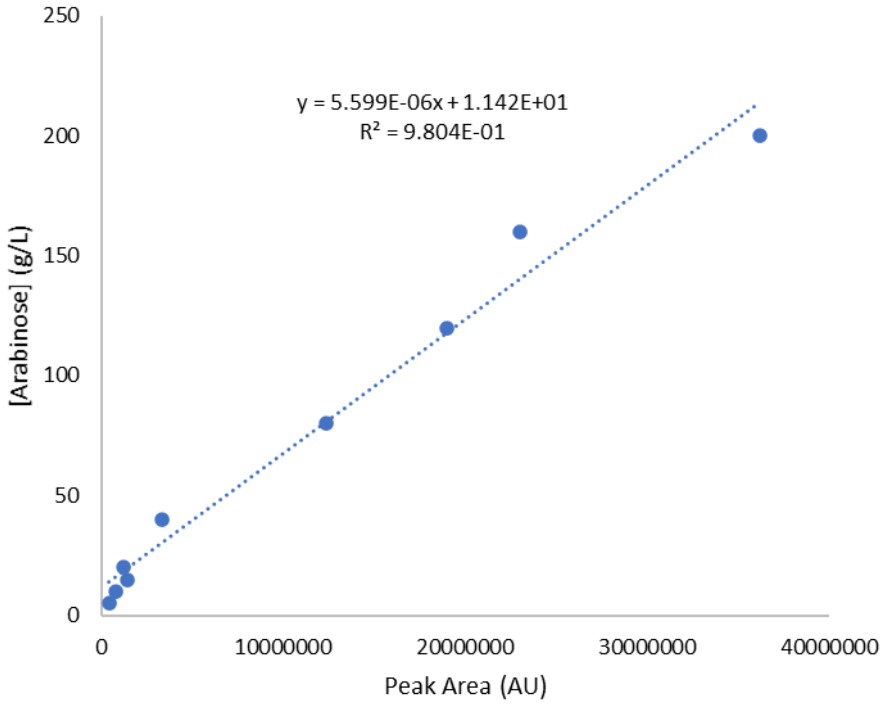


Figure A1 – Arabinose calibration curve in HPLC. Arabinose was determined by refraction index detection and the peaks area are expressed in arbitrary units (AU). The obtained calibration rate is observed in the figure.

Annex 2.1. – Logarithmic *E. coli* growth curves for wild type strains, and strains with the pMLBAD and pSAS39 plasmid, in different conditions of growth and with or without induction of antisense production.

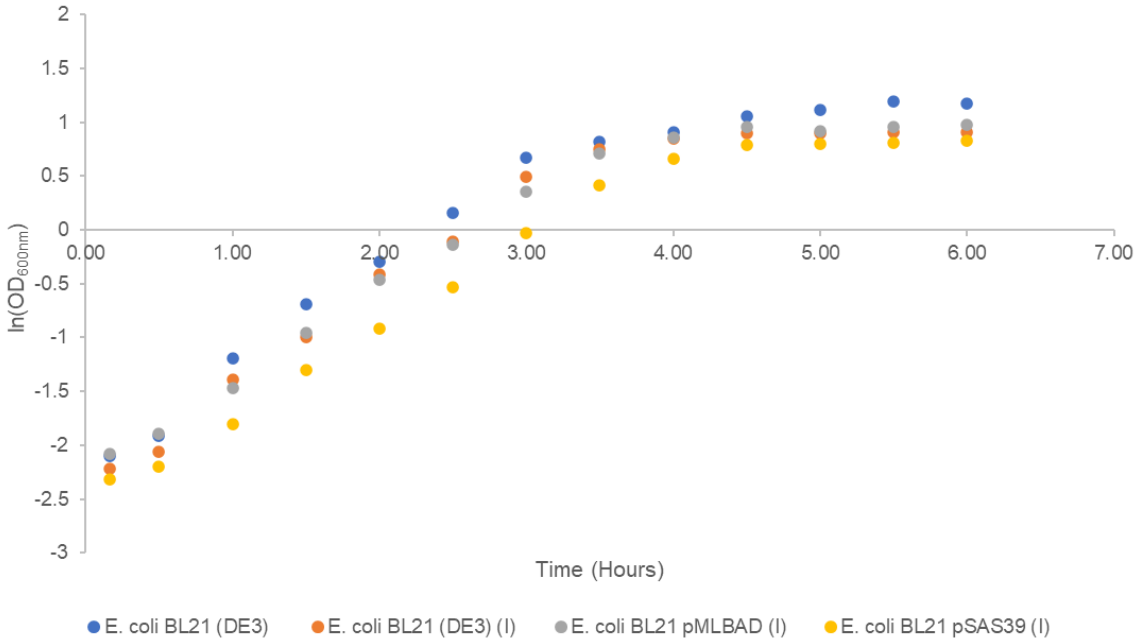
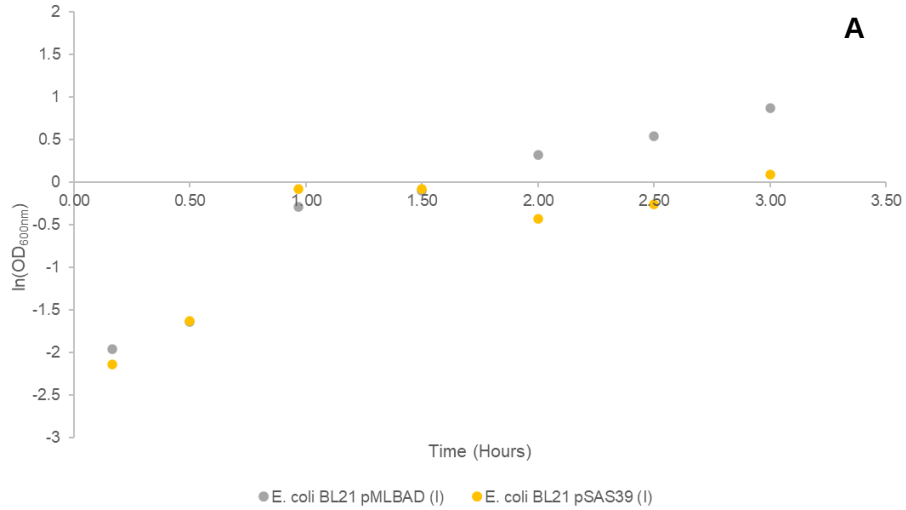


Figure A2.1. – Logarithmic growth curves for *E. coli* BL21 (DE3) wild type, with and without induction (in orange and blue, respectively), *E. coli* BL21 (DE3) with the pMLBAD plasmid (in gray) and *E. coli* BL21 (DE3) with the pSAS39 plasmid (in yellow). The growth was conducted in LB liquid medium at 37 ºC and 250 rpm. (I) – Indicates that cells were induced with L-arabinose 2 % (w/v).

Annex 2.2. – Logarithmic *E. coli* growth curves with the pMLBAD and pSAS39 plasmid, in different conditions of growth and with induction of antisense production.



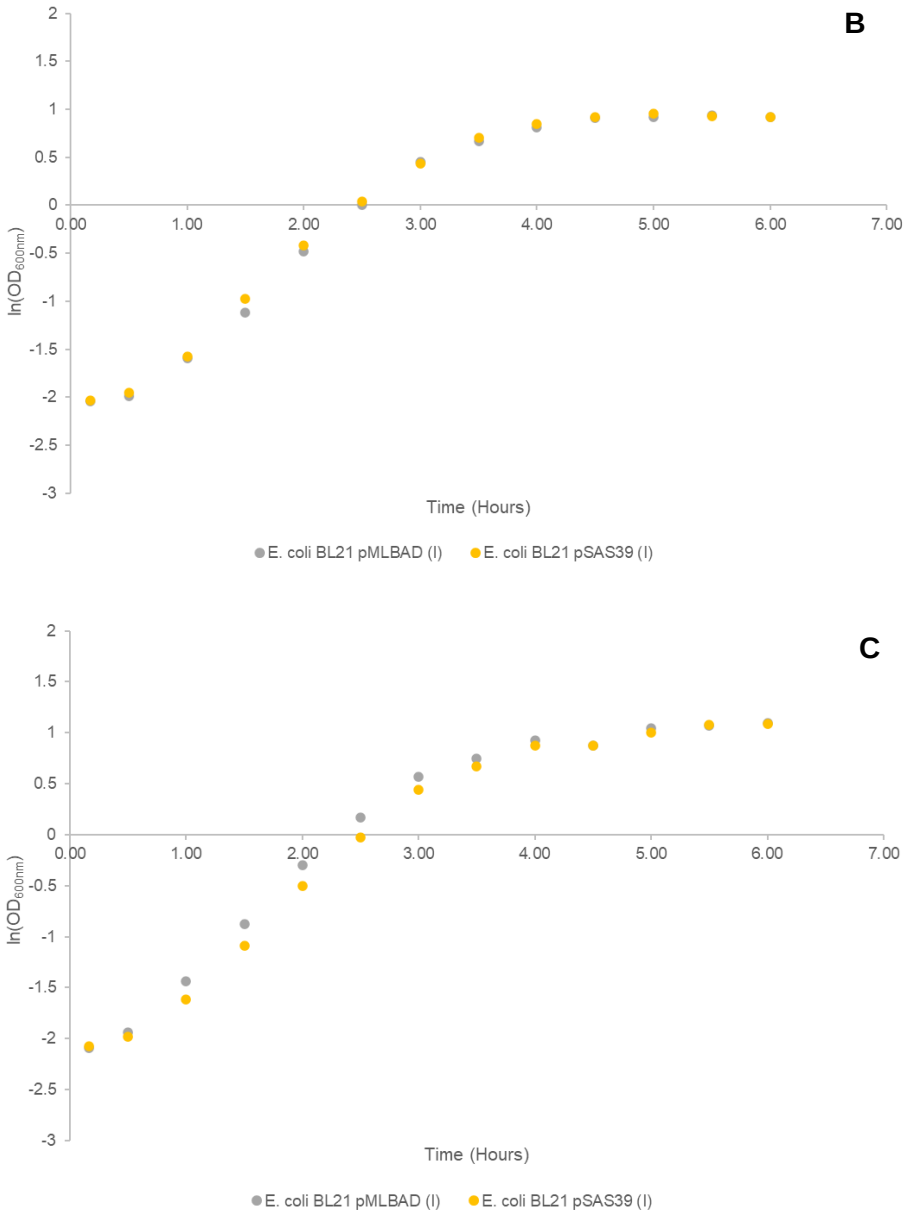


Figure A2.2. – Logarithmic growth curves for *E. coli* BL21 (DE3) with pMLBAD or pSAS39, with induction. *E. coli* BL21 (DE3) with the pMLBAD plasmid (in gray) and *E. coli* BL21 (DE3) with the pSAS39 plasmid (in yellow). The growth was conducted in LB liquid medium at 37 ºC and 250 rpm. (I) – Indicates that cells were induced with L-arabinose 2 % (w/v). (A) – cell culture with an intermediate culture induced at T0; final culture induced again at T0 and T2. (B) – cell culture with induction at T3. (C) – cell culture with induction at T4.