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Development of an Escherichia coli strain to express bacterial antigenic peptide and co-express Braun's lipoprotein antisense oligonucleotide

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Abstract

Aquaculture is a really important sector of the food industry. Through the fact that fish in such cultures live together very close, it is super easy for diseases like Vibriosis to transmit between them. Therefore, it is important to develop vaccines for the fish against such diseases. Thus, the aim of this study was to overexpress the OmpK protein, which is widely distributed on the cell envelope of *Vibrio* species, in a *Escherichia coli* industrial strain, so that, once purified, an antibody can be raised for fundamental studies and OmpK production optimized to be used as vaccine against Vibriosis.

In the beginning, OmpK produced from other recombinant *E. coli* cells that were obtained from a previous study was overexpressed. Although, the overexpression was successful, a purified soluble protein extract revealed high endotoxins content, and so that protein solution could not be used for the production of the antibody. A HIC was carried out to see, if that way the endotoxins could be separated from the protein efficiently. The chromatogram showed two isolated peaks which may indicate a sufficient separation. As the concentration of the OmpK protein was too low for a confirmation of this result, this part has to be carried out again with a higher concentrated protein solution.

The first main part of this thesis was to build a plasmid with the sequence of a Lpp antisense, with the objective of increasing the outer membrane permeability of the expressing cells. After the cloning steps in *E. coli* αDH5, which obtained the pMLBAD vector with the sequence for the Lpp antisense, the plasmid was transformed into *E. coli* BL21 (DE3) and some physiological tests were carried out to check the effect of the plasmid on the cells. None of the tests showed the expected results. Just afterwards the result of the sequencing arrived, which revealed that the insert in the plasmid was not the desired one.

The second main part was to express the outer membrane protein itself. As for the production of antibodies it is not necessary to use the whole protein but an antigenic fragment of it, just the N-terminal part of OmpK with the natural signal peptide was cloned. When the pET23a+ vector containing the correct sequence was transformed into *E. coli* BL21 (DE3), several different attempts of overexpression were carried out.

Unfortunately, none of them was successful and different attempts of the overproduction of the protein still have to be tried.

Keywords: aquaculture, fish vaccine, OmpK, recombinant protein

Table of content

List of abbreviations

AMP	-	adenosine monophosphate
Amp	-	ampicillin
APS	-	ammonium persulphate
araC	-	arabinose operon regulatory protein
bp	-	base pair
BSA	-	bovine serum albumin
CAP	-	catabolite activator protein
CFU	-	colony forming unity
CV	-	column volumes
dhfr	-	dihydrofolate reductase
dNTP	-	deoxyribonucleotide triphosphate
DTT	-	dithiothreitol
ECL	-	enhanced chemiluminescence
EDTA	-	ethylenediaminetetraacetic acid
EtBr	-	ethidium bromide
HF	-	High-Fidelity
HIC	-	hydrophobic interaction chromatography
IMAC	-	immobilized metal affinity chromatography
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
Kdo	-	2-keto 3-deoxy-octulosonate
lac	-	lactose
LB	-	Lennox broth
LOS	-	lipoolygosaccharide
Lpp	-	Braun's lipoprotein
LPS	-	lipopolysaccharide
mob	-	mobilization region
NC	-	nitrocellulose
NCBI	-	National Center for Biotechnology Information
OD	-	optical density
Omp	-	outer membrane protein
OPS	-	O-polysaccharide
ORF	-	open reading frame
ori	-	origin of replication
PBS	-	Phosphate-buffered saline

PCR	-	polymerase chain reaction
Pred-TMBB	-	Prediction of TransMembrane Beta-Barrel Proteins
Rep	-	replication protein
SB	-	Super broth
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TEMED	-	Tetramethylethylenediamine
TEV	-	tobacco etch virus
TCM	-	Tris-Calcium-Magnesium
Tris	-	tris(hydroxymethyl)aminomethane
tp	-	trimethoprim

Previous work

Basis of this work is the thesis of Fiúza Almeida Lopes (2017). He worked on the use of immunogenic *Vibrio alginolyticus* OmpK as template for the production of antibodies and so the development of a vaccine to protect fish cultures from *Vibrio* infections. For this purpose he amplified the *Vibrio alginolyticus* gene encoding OmpK, cloned it into pET23a+ and transformed it into *E. coli* BL21 (DE3). With these cells he overproduced the recombinant OmpK and purified it.

As Fiúza Almeida Lopes (2017) had problems with the purification of the protein, the hypothesis was made to overexpress a soluble truncated OmpK 1-99 fragment, which should be easier to express and purify. One problem was the removal of endotoxins from the proteins. To find a solution for this, it was tried to separate the protein and the endotoxins by HIC.

Besides the overproduction of the truncated OmpK 1-99 and the purification of the protein from endotoxins, the third part of this thesis was the co-expression of Lpp antisense mRNA to facilitate the secretion of the recombinant protein. These three parts are elucidated below in a general introduction for all parts, separated methods, results and discussion and in the end a general conclusion plus some future perspectives.

1. Introduction

1.1 Aquaculture

In recent years the aquaculture industry developed to an important source of seafood. The agriculture of the oceans is growing a lot faster than the sectors which produce other food animals. This is due to the fact that the capture fishing industry gets less and wild stocks get smaller. As the fish in the aquaculture live pretty close together, it is easy for infectious disease agents to be transmitted between either single or even multiple species. Some decades ago, fish were not vaccinated at all. If a disease like vibriosis affected the fish population, a big amount of antibiotics was needed to prevent the industry to crash completely. In the 1970s, fish immersion vaccines, which were based on formalin-inactivated broth cultures, showed a positive effect against vibriosis and soon similar vaccines were developed to fight also other *Vibrio* diseases. Through the use of the vaccines, the use of antibiotics dropped significantly and fish production increased. For an optimal effect of vaccines, but also for prophylaxis of infectious diseases, it is important to maintain proper fish management with good hygiene and limited stress. As the development of human and veterinary vaccines is an ongoing interaction of different factors, also the development of fish vaccines comprises of interplay between academia, the pharmaceutical industry and regulatory authorities (Sommerset et al., 2005).

1.2 Vibriosis

The genus *Vibrio* are gram-negative bacteria, often in the shape of rods with the ability to move, mesophilic and chemoorganotrophic. Their metabolism is facultative fermentative and according to their 16S rRNA they are classified as *Gammaproteobacteria*. The preferred habitats of these bacteria are aquatic where they are associated with eukaryotes (Thompson et al., 2005). Presently, 46 species are known to belong to the genus *Vibrio*. Even when most of them are considered to be harmless to humans, some of them are potentially dangerous. 12 species are linked with some human infections like gastroenteritis, localized wound infections and systemic infections which result in bacteraemia and septicaemia. The majority of *Vibrio* species is known to be associated with fish and shellfish (Li et al., 2010b).

Vibrio harveyi, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* are pathogens which cause vibriosis. This infection affects marine fish in tropical and subtropical zones (Li et al., 2010a) and causes a substantial limitation on the industry (Ningqiu et al., 2008). Fish which are affected with vibriosis typically do not eat well, are lethargic, have a dark colour and often stay close to the surface (Bruno et al., 2013). Further symptoms can be tissue and appendage necrosis, body malformation, bolitas negricans, bioluminescence, muscle opacity, anorexia and their growth and larval metamorphosis is slow (Aguirre-Guzman et al., 2004). *Vibrios* are often opportunists, which means that they only cause a disease when the host organism has physiological stress like for example immune suppression. Therefore infection occurs more often in intensive cultures and in adverse environmental conditions (Defoirdt et al., 2007).

One attempt to prevent the fish population from bacterial diseases is the use of antimicrobial substances, but this has some drawbacks like pathogenic strains which get a resistance or also problems with the food safety. An alternative, and also safer, strategy is the use of vaccination. Both strategies, to use whole cells or just cell components as immunogens, have been already tested (Ningqiu et al., 2008).

1.3 OmpK

As the outer membrane proteins (OMPs) of the species *Vibrio* have exposed epitopes on the surface of the cell, they are highly immunogenic components (Li et al., 2010b). One of these outer membrane proteins, which is likely to be an immunogenic antigen for some *Vibrio* species, is OmpK (Ningqiu et al., 2008). The OmpK gene of *Vibrio* consists of 789 nucleotides which encode 263 amino acids. The first 20 amino acids build up the signal peptide, which means that the mature OmpK consists of the remaining 243 amino acids. This protein has a calculated molecular mass of 27,458 Da (Inoue et al., 1995b).

Recombinant OmpK was used as a vaccine for Orange-spotted groupers (*Epinephelus coioides*) and it challenged with virulent *V. harveyi*. With this study the protective properties of OmpK were documented. Further, the large yellow croakers (*Pseudosciaena crocea*) were vaccinated with OmpK of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*. Thereafter, the fish showed a high protection against the

infection by the pathogens. No sequence variations of *ompK* genes in *Vibrio* species have been reported yet and it is supposed that this outer membrane protein is a shared antigen among *Vibri*os (Ningqiu et al., 2008).

Inoue et al. (1995a) reported that OmpK works as the receptor of the virulent vibriophage KVP40. This vibriophage is a member of the family *Myoviridae* and has been isolated with *Vibrio parahaemolyticus* 1010 as host. At least 8 *Vibrio* and one *Photobacterium* species can work as the host of KVP40 which indicates that they have to have a common cell surface component that serves as receptor for the phage.

Fiúza Almeida Lopes (2017) obtained in his thesis a 2D image of the OmpK protein structure using Pred-TMBB. In Figure 1 regions that interact with the extracellular space can be seen. OmpK shows 4 highly immunogenic regions that can be used for interaction with antibodies.

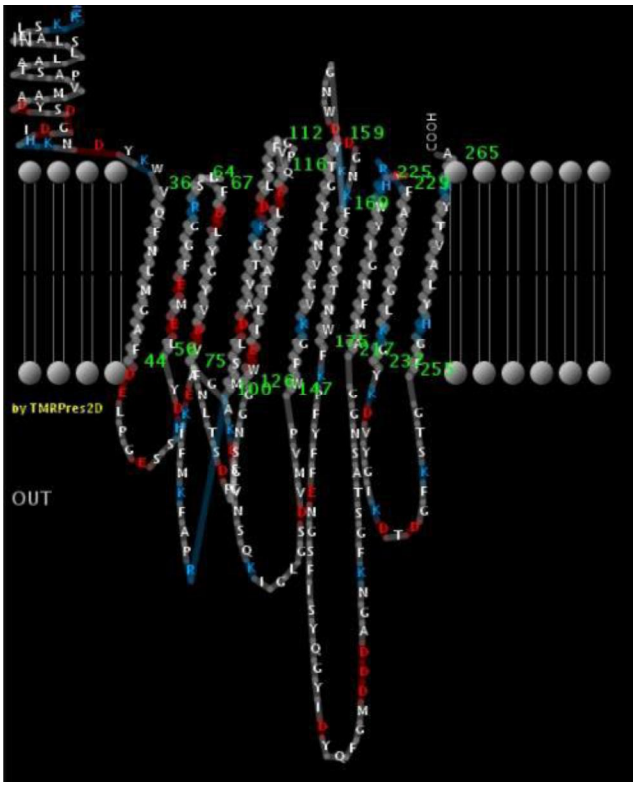

Figure 1: 2D image of the OmpK structure predicted by Pred-TMBB (Fiúza Almeida Lopes, 2017)

1.4 Braun's lipoprotein (Lpp)

To ensure that the structural integrity of the bacterial cell envelope is maintained, the outer membrane of gram-negative bacteria consists of many different proteins. Some of them have an added lipid, N-acyl-S-diacylglyceryl cysteine and are called lipoproteins. Lpp is an abundant protein, which is associated with the outer membranes of *Enterobacteriaceae*. This protein connects the murein (peptidoglycan) layer with the outer bacterial membrane (Sha et al., 2008) through not just covalent, but also non-covalent mechanisms and so it makes the cell envelopes rigid (Ni et al., 2007). It is known that Lpp plays a role in the immune response of the host against infections with gram-negative eneric pathogens like *E. coli*, *Salmonella enterica serovar typhimurium* and *Y. enterocolitica* (Sha et al., 2008). In *E. coli* the Lpp have about 700,000 copies per cell, being the most abundant protein (Ni et al., 2007).

Lpp is trimerized and the crystallographic structure of the protein has been solved already (Cascales et al., 2002). It is suggested to consist of oligomeric α-helices and it works as a barrier against metabolites and antibiotics (Chang et al., 2012). About one-third of the lipoprotein is bound to murein in a covalent way. If Lpp is absent or there is a mutation in the gene which affects the covalent attachment to the murein layer, the outer membrane forms bubbles. In this case cells become hypersensitive to some toxic compounds and they release proteins from the periplasm to the extracellular medium (Cascales et al., 2002).

1.5 Antisense Oligonucleotides

In the concept of antisense oligonucleotides, sequences are used which are complementary to the Watson-Crick base pair (bp) hybridization. These fragments can inhibit the expression of a specific mRNA and so the translation to protein is blocked (Dias and Stein, 2002). If a protein is targeted that is necessary for cell growth or also intracellular viral replication, the antisense agent will cause a slowdown of these processes (Stein and Cheng, 1993).

Two classes of antisense oligonucleotides can be distinguished: (a) the ones which induce the degradation of mRNA, which are the RNase H-dependent oligonucleotides and (b) the ones which prevent or inhibit the progression of splicing or the translational machinery physically, which are the steric-blocker

oligonucleotides. The ubiquitous enzyme RNase H performs a hydrolysation of the RNA strand of the RNA/DNA construct. One advantage of RNase H-dependent oligonucleotides is that they can be targeted to any region of the mRNA and thus inhibit protein expression. In contrast, steric-blocker oligonucleotides have to be targeted to the 5'- or start codon region to be properly efficient. About 70 % of all oligonucleotide sequences are targeting the translation initiation codon, but this region is not always the best option. Sites that can be used as target as well are sites in the 3'- or 5'- untranslated region. A less targetable region is the coding region itself. Just in a few cases the coding region can be targeted properly. This could be due to the fact that the oligonucleotide can be untwined from the targeted mRNA by the ribosomal machinery (Dias and Stein, 2002).

Recent work with the antisense technology was carried out by Watanabe et al. (2018) in the topic of Duchenne Muscular Dystrophy, Goemans et al. (2018) who use antisense oligonucleotides in Drisapersen, a drug for the same disease or also Ämmälä et al. (2018) who deliver targeted antisense oligonucleotides to pancreatic β-cells what broadens the use of antisense oligonucleotides for future studies.

1.6 Expression systems

For heterologous protein production mostly bacterial expression systems are used. This is due to their rapid growth, the growth at high cell density on cheap substrates, the amount of information that is already known of their genetics and the high number and availability of cloning vectors and mutant host strains. For high production levels of protein it is recommended to clone the gene downstream of regulated promoter which is well-characterized. The choice of which host and promoter system should be used depends on the heterologous protein which is produced. If posttranslational modifications are crucial for the function of the protein, bacterial expression systems should not be chosen, but yeasts, filamentous fungi or insect and mammalian cell cultures can be used for that task (Rosano and Ceccarelli, 2014).

1.6.1 *Escherichia coli*

For the heterologous protein production the most commonly used organism is the gram-negative bacterium *E. coli*. This organism is very well-known and established in every laboratory and most commonly used for pharmaceutical and industrial protein production, as well as for large-scale production. One drawback of the use of *E. coli* for the production of recombinant proteins for therapeutic use is that this organism accumulates lipopolysaccharides (LPS). These also called endotoxins are pyrogenic in humans and other mammals and therefore they have to be removed in a cascade of purification steps. The accumulation of the overexpressed recombinant proteins can happen either in the cytoplasm or periplasmic space. As in the cytoplasm a higher yield can be achieved, heterologous proteins are most frequently overexpressed there. Often used *E. coli* strains are BL21 and K12 and their derivatives. The difference between K12 and BL21 is that the latter one is *lon* and *ompT* protease deficient. The overexpression of a gene in a foreign host faces often a lot of problems. An example is the difference of the codon usage between *E. coli* and the heterologous protein, especially rare codons. Rare or lacking of needed tRNAs in the expression host can lead to translational stalling, premature translation termination, translation frameshift and incorporation of wrong amino acids. One attempt to decrease this problem is to modify culture conditions, like lowering the temperature or some changes in the media composition. A further possibility is to supply the abundant amino acid in the culture medium. There are some *E. coli* strains which supply additional tRNAs under control of their native promoters. Expression problems can also be reduced by the choice of the copy number of the plasmid. If the recombinant protein is toxic for the host, then it is better to use a low copy number vector. Another drawback of the overexpression of proteins in the cytoplasm is that they can form inclusion bodies. This can be counteracted by lowering the temperature, substitution of amino acids, co-expression of chaperones, changing culture conditions like the pH or change of the bacterial strain. An alternative to that is to solubilize and refold the inclusion bodies to get active and functional proteins. One *E. coli* strain that is especially designed to overexpress membrane proteins is BL21 (Terpe, 2006). This is reasoned by the deficiency in the Lon protease, through which many foreign proteins are degraded and the missing of the gene which encodes the outer membrane protease OmpT. The function of this protease is the degradation of extracellular proteins. These mutations are favourable for the expression of

recombinant proteins as after cell lysis to release the proteins, these may be digested by OmpT (Rosano and Ceccarelli, 2014).

To simplify the downstream and N-terminal procession of overexpressed proteins by facilitating their correct folding, especially proteins with disulphide bonds, they are exported from the bacterial cytoplasm to the periplasm. For this secretion process a signal peptide has to be present at the N-terminus. If the wanted protein is located in the periplasm it can be easily released into the culture medium. This can be carried out by osmotic shock or cell wall permeabilisation, so that cell lysis is not necessary and there is no contamination of the target protein with cytoplasmic proteins during the purification process. There are several problems that can lead to insufficient production of the heterologous protein in the periplasmic space: incomplete translocation across the inner membrane, proteolytic degradation and lacking capacity of the export machinery. By careful balancing of the promoter strength and gene copy number the expression level can be optimized (Terpe, 2006).

Already some decades ago *E. coli* was intensively used for the production of recombinant biopharmaceuticals like Humulin for the treatment of diabetes, Protropin which is used from patients with hGH deficiency or also IntronA to treat cancer, genital warts and hepatitis, all approved in the 1980s. Even when lately also other cell factories like mammalian cells or *S. cerevisiae* are used more and more for the production of recombinant proteins, more than two third of recombinant biopharmaceuticals against cancer are produced in *E. coli (Sanchez-Garcia et al., 2016)*. In recent years, *E. coli* was used as the host organism e. g. for the production of recombinant swollenin from *Trichoderma harzianum (Santos et al., 2017)*, scaled-up production of soluble, disulphide bonded proteins in the cytoplasm of *E. coli* at high cell densities and protein yields (Gaciarz et al., 2017) or also improved recombinant membrane protein production achieved by combination of transcriptional tuning and codon usage algorithms (Claassens et al., 2017).

1.6.2 *Vector systems*

Rosano and Ceccarelli (2014) depict a good overview of the necessary features of an expression vector (Figure 2).

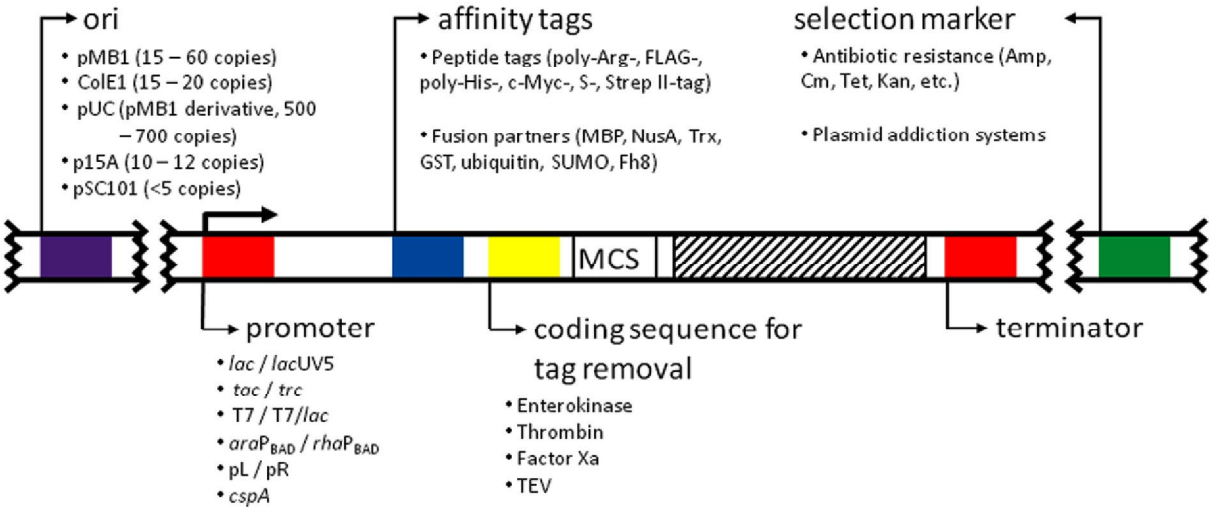

Figure 2: basic composition of an expression vector (Rosano and Ceccarelli, 2014)

To achieve a selection between cells with the desired plasmid and plasmid-free cells, the plasmid backbone carries a selection marker. In most cases this selection marker is an antibiotic resistance gene. The often used resistance to ampicillin is encoded by the *bla* gene. This gene is expressed as β-lactamase which inactivates the β-lactam ring of β-lactam antibiotics. One drawback of this periplasmic enzyme is that it is secreted continuously, so it can be degraded and the ampicillin is almost depleted in some hours. If that is the case, cells which do not carry the plasmid are allowed to grow. An alternative to these kinds of antibiotics is tetracycline. This antibiotic has a different mechanism, as the antibiotic is flowing out of resistant cells actively. As the use of antibiotics is quite expensive and resistances of antibiotics can occur, their use is not favourable for large-scale cultures. Because of these reasons antibiotics-free plasmid systems have been developed. These systems rely on plasmid addiction, what means that cells which do not contain the plasmid are not able to grow or live. This can be achieved by deletion of an essential gene from the bacterial genome and placing it on the plasmid. There are three different types of plasmid-addiction systems: toxin/antitoxin-based systems, metabolism-based systems and operator repressor titration systems (Rosano and Ceccarelli, 2014).

Affinity tags are used to detect the expressed protein along the expression and the purification. By using them, maximal solubility can be achieved and the protein can be purified from the cell broth. For this purpose either a peptide tag or a large polypeptide can be fused with the desired protein. The use of small peptide tags interferes less, but sometimes they can have a negative effect on the tertiary structure or also the biological activity of the protein. Such tags can be positioned either on the N-terminal or the C-terminal end. If a tag is added, the recombinant protein can be detected with the use of commercial antibodies to detect the protein along expression trials, even if their concentration is very low. Using an affinity tag, the protein can be easily purified with resins that bind to the tag tightly and specifically. Often, proteins are His-tagged and so they can be recovered with nitrilotriacetic acid-agarose resins loaded with Ni^{2+} or Co^{2+}. If enhanced solubility of the protein is aimed, non-peptide fusion partners can be added. Examples for these are the maltose-binding protein (MBP), N-utilization substance protein A (NusA), thioredoxin (Trx), glutathione S-transferase (GST) and ubiquitine. If the affinity tag has to be removed (e. g. for structural or biochemical studies on the recombinant protein) this can be achieved by enzymatic or chemical cleavage. When enzyme digestion is the chosen method, the expression vector must have the sequence for protease cleavage sites downstream of the tag-gene. Enzyme that can be used for that task are enterokinase, thrombin, factor Xa and the tobacco etch virus (TEV) protease. Due to the high specificity of TEV, the easy production and the fact that it leaves just a serine or glycine residue, it is often used for the removal of His-tags. One example for a chemical reagent that is used to remove tags is cyanogen bromide (DNBr). However, as the chemical removal often causes unwanted protein modification and the reaction conditions are harsh, this method is not the best choice (Rosano and Ceccarelli, 2014).

If more than one vector should be used in the same host cell these must belong to distinct incompatibility groups. This is due to the fact that they would compete with each other for the replication machinery. So if the first vector is either of the pMB1 origin or mutated version thereof (pET, pUC or pQE series), systems with the p15A ori (pACYC and pBAD series) can be used for the dual expression. For the rare case that a triple expression is needed, the pSC101 can be used as the third system (Rosano and Ceccarelli, 2014).

1.6.2.1 pET-23a

In Figure 3, Novagen (2018a) shows the backbone of the pET-23a(+) expression vector with its main features.

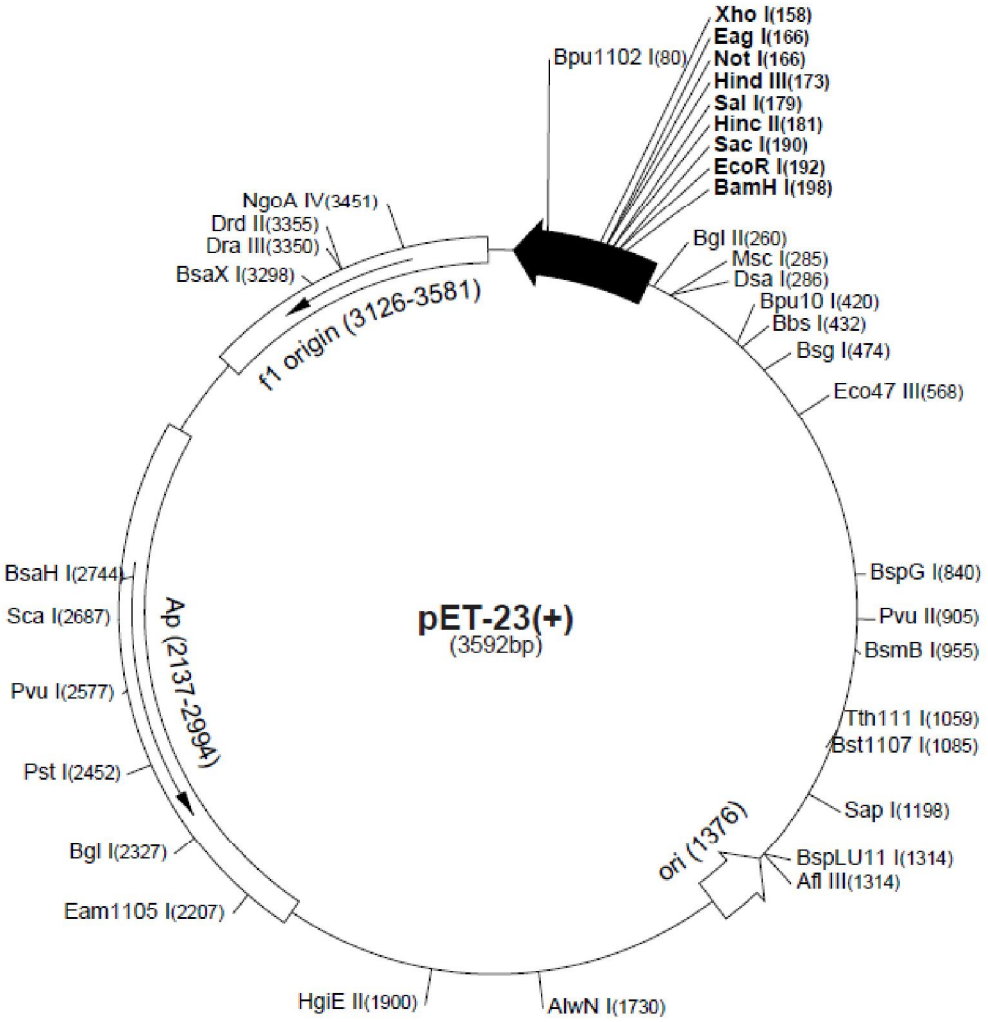

Figure 3: backbone of the pET-23a(+) vector (Novagen, 2018a)

pET is one of the most frequently used vector systems. In particular, pET23a+ is a bacterial plasmid with the size of 3666 bp. Some properties which make it such a popular vector are the multiple cloning site (BamHI to XhoI), F1 phage origin for ssDNA production and that it is easy to handle. The plasmid has its origin in *E. coli* (pBR322-type ColE1), it contains an ampicillin resistance gene (β-lactamase) and as it has a T7 promoter it can be induced by IPTG in an indirect way. It has sequences for a His tag C-terminal fusion with the cloned protein coding region (Novagen, 2018a). The copy number of ColE1-derivatives is about 15 – 60 copies per cell (Rosano and Ceccarelli, 2014).

By using the T7 RNA polymerase instead of the normal *E. coli* RNA polymerase, the chains are elongated about five times faster. The chromosome of *E. coli* BL21 (DE3) contains the gene for the T7 polymerase. A *lac* promoter derivative, L8-UV5 *lac,* controls the expression of that gene. This promoter contains point mutations which distinguish it from the wild-type *lac* promoter. The -10 region contains two of these point mutations, which lead to an increased promoter strength and a decrease in dependency on cyclic AMP and CAP - Its receptor protein. Through a third point mutation the promoter is less sensitive to glucose, which allows IPTG to induce the T7 RNA polymerase, even if glucose is present (Terpe, 2006).

1.6.2.2 pMLBAD

Lefebre and Valvano (2002) constructed some vectors which can be used for gene expression in *Burkholderia cepacia*. They started with a derivative of the pBBR1 plasmid, the pME6000, which was initially isolated from *Bordetella bronchiseptica*. This plasmid is maintained at about 20 to 30 copies per cell. The main features of the vector can be seen in Figure 4. One coding region of the vector represents a replication protein (Rep) which is homologous with replication proteins in gram-negative bacteria. This protein is important for constant plasmid maintenance, even when antibiotic selection is absent. This is due to the fact that the original pBBR1 plasmid was maintained in *B. bronchiseptica* in a natural way without any known selectable markers. A further region that is important on the pBBR1 is the mobilization (mob) region which encodes a putative protein that is homologous to Mob/Pre proteins of Gram-positive bacteria. When transfer functions are supplied in *trans*, this region is essential for the mediation of plasmid transfer. The arabinose inducible BAD promotor sequence was placed directly upstream of a multiple cloning site combined with the previously mentioned minimal sequence required for plasmid maintenance and mobilization. If L-arabinose is present, it activates, together with the regulatory protein AraC, the transcription from the P_{BAD} promoter. One further function of AraC is that if glucose is present, it acts as a transcriptional repressor and so prevents the transcription from the P_{BAD} promoter. Therefore, this is a tightly regulated system for gene expression. To obtain a selectable marker, the *dhfr* gene is placed on the vector which encodes resistance to trimethoprim. Downstream of the multiple cloning site, the strong ribosomal *rrnB* transcriptional terminator from

pBAD24 is encoded. The function of this gene is to stop the transcription before it comes to the vector backbone.

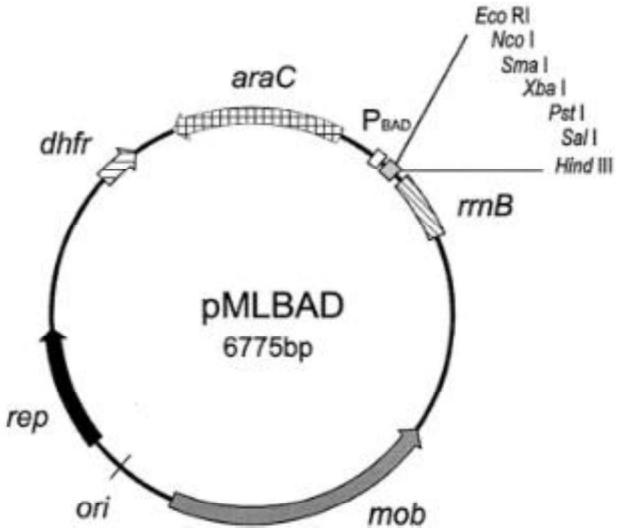

Figure 4: backbone of the pMLBAD expression vector (Lefebre and Valvano, 2002)

1.6.3 *Endotoxins*

Since more than 100 years it is known that some bacterial products have the ability to affect humans and other animals. One class of bacterial products are recognized to be associated closely with the bacterium and they are released just during bacterial lysis. During cell lysis, these toxic compounds are excreted into the surrounding medium. To distinguish them from the toxic material that is synthesized and excreted by intact bacterium, which are called "exotoxins", these are named "endotoxins". Endotoxins have a broad spectrum of biologic activities. Gram-negative bacteria are the only ones to produce true endotoxins and they are part of the bacterial cell wall (Morrison and Ulevitch, 1978).

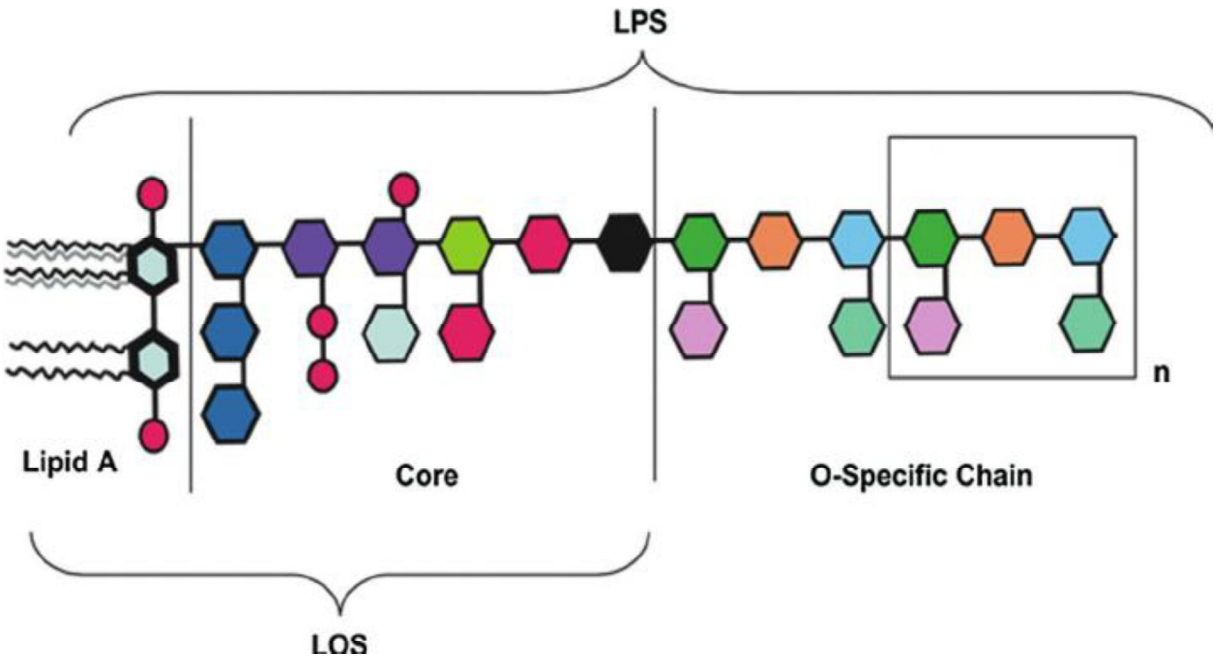

Figure 5: General chemical structure of LPS in Gram-negative bacteria. The polysaccharide moiety (LPS) consists of the core region and an O-specific chain. The blue parts next to the Lipid A represent the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) and the violet ones the heptose residues. Phosphate residues are shown as pink appendages (Silipo et al., 2010)

All gram-negative bacteria have a similar lipopolysaccharide (LPS) structure with flexible amounts of polysaccharide and lipid. Basically it is a glycol-conjugate which consists of the lipid moiety lipid A, which is anchored to the membrane by fatty acids and is linked to a saccharide part in a covalent way. In this way it is distinguished from lipids which are associated with the LPS molecule and bound in a noncovalent loose way. The part of the LPS molecule, which consists of polysaccharide can be distinguished in two regions: an oligosaccharide (core region) and a polysaccharide moiety (OPS, O-polysaccharide). The core structure of the core polysaccharide region is defined by a unique deoxysugar 2-keto-3-deoxy-octulosonate (Kdo), which anchors the core region to the lipid A, heptose, phosphorylethanolamine and several hexoses. This region is often identical for large groups of bacteria. The second region, the O-antigen is different. It is chemically unique for each type of LPS and organism. These antigens consist of repeating oligosaccharide units, where each contains three to four different hexose units. The amount of the repeating oligosaccharide units can vary between two and ten, even in the same bacterium. Depending on whether the OPS is present or not, it determines if the bacterial colony

appears smooth or rough. Rough ones are also called lipo-oligosaccharide (LOS) containing cell colonies. As more than a hundred different monosaccharides plus many types of noncarbohydrate substituents (acyl, amino acid, hydroxyl acid, alkoxy, keto acid groups) have been found already in the OPSs, they are the most variable domain of LPSs (Silipo et al., 2010).

In the outer membrane of Gram-negative bacteria the LPS reduces the cell envelope permeability. Because of this barrier function, bacteria are able to grow in unfavourable conditions. If bacteria have a defect in the *lps* gene, they are more sensitive to antibiotics and antimicrobial peptides. As the LPS is located on the outermost surface of the cell, it can highly interact with the surrounding environment. Gram-negative organisms are often a major constituent of the resident flora of mammalian species. Therefore they interact with the intestinal tract of them (Morrison and Ulevitch, 1978).

Galanos et al. (1985) indicate fever, lethality, Shwartzman reactivity, macrophage, B-lymphocyte activation and other activities as acute pathophysiological effects of endotoxins. The endotoxic properties of LPS are induced by the lipid A, which is the active centre. The polysaccharide portion of the molecule is not necessary for the toxicity. During an infection, the endotoxin is released from the outer cell membrane of Gram-negative bacteria. The result is a serious bacterial sepsis which causes death in over 40 % of cases (Frecer et al., 2000).

1.7 Hydrophobic Interaction Chromatography (HIC)

When HIC media is used, the separation of biomolecules (here proteins in particular) during the chromatographic process relies on interplay between the hydrophobicity of the stationary phase, the composition and nature of the sample, the amount and distribution of surface-exposed hydrophobic amino acid residues and the concentration and type of salt in the binding buffer. HIC is closely related to reversed phase chromatography (RPC), with the difference that the adsorption of biological solutes to HIC adsorbents is promoted or modulated when relatively high concentrations of kosmotropic (anti-chaotropic) salts (e. g. ammonium sulphate, sodium sulphate) are present. To loosen the bound solutes, a stepwise or gradient elution with buffers of low salt content is carried out. Hydrophobic adsorbents are

based on alkyl or aryl ligands which are attached to a hydrophilic base matrix like Sepharose. To adapt the characteristics of the matrix to the hydrophobicity of the proteins (weak, moderate or strong), the type and concentration of the ligand is optimized (GE Healthcare Bio-Sciences, 2014).

1.7.1 Factors affecting HIC

There are several parameters which have to be considered for the selection of a HIC medium.

- type and concentration of salt
- structure and concentration of the ligand
- the nature of the base matrix (e. g. agarose, organic copolymers)
- characteristics of the target protein and other sample components
- temperature
- pH

The first two parameters are the most important ones in terms of determination of the outcome of a HIC event. The selectivity of the adsorption is determined by the type of immobilized ligand whereas the adsorption capacity is determined by the concentration of it. One of the most important parameters, which have an influence on the capacity and the selectivity in HIC, is the solvent. The start buffer has to be optimized according to pH, type of solvent and type and concentration of the salt. When "salting-out" salts are added to the sample, the ligand-protein interactions in HIC are enhanced. Through the higher concentration of salt, the amount of bound protein goes up to the precipitation point of the protein. $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl and CH_3COONH_4 are the most commonly used salts (GE Healthcare Bio-Sciences, 2014).

2. Material and Methods

2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this work are listed in Table 1.

Table 1: strains and plasmids used in this study

Strain/Plasmids	Description	Source
	***E. coli* strains**	
αDH5	supE44 (ϕ80 lacZΔM15) hsdR17(rK - mK +) recA1 endA1 gyrA96 thi-1 relA1 deoR Δ(lacZYA-argF)U169	Invitrogen
BL21 (DE3)	FompT hsdSB (rB - mB -) dcm gal λ(DE3)	Stratagene
	Plasmids	
pET23a+	cloning vector, Ap	Novagen
pJBF2	pET23a+ containing the ompK gene, Ap	Fiúza Almeida Lopes (2017)
pMLBAD	cloning vector	Lefebre and Valvano (2002)
pAR1	pET23a+ containing truncated OmpK 1-99 sequence	this thesis
pAR2	pMLBAD containing *E. coli* Lpp antisense sequence	this thesis

When in use, *E. coli* strains were maintained in Lennox broth (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g agar), supplemented with 150 μg/mL of ampicillin (Ap) or 100 μg/mL of trimethoprim (Tp), when required.

Unless otherwise mentioned, the liquid culture were carried out in LB liquid medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with the appropriate antibiotics at 37 °C, with 250 rpm of orbital agitation. Bacterial growth was followed by measuring the culture optical density at 640 nm (OD_{640}) in a spectrophotometer (Hitachi U-2000).

2.2 Overexpression of the whole OmpK

50 mL of LB liquid medium containing 150 µg/mL ampicillin was preinoculated with a colony of *E. coli* BL21 (DE3) containing the pJBF2 vector and grown under the conditions as described above for at least 3 hours. The OD_{640} of the bacterial culture was determined and an appropriate aliquot of preinoculum was used to get an initial OD_{640} of 0.1 in 100 mL SB liquid medium (32 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl) (2 X 200 mL in the second batch) supplemented with 150 µg/mL ampicillin. The bacterial culture was incubated at 30 °C with 250 rpm orbital agitation. When the cultures reached an OD640 of around 0.5 (time T0) the lacUV5 promoter was induced with 0.4 mM IPTG. Then, the culture was further incubated 16 hours at the same conditions (time T16).

For sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis the amount of sample that was taken from the batch corresponded to 1 mL of sample with OD_{640} of 0.6. Then, the sample was centrifuged 5 minutes at maximal speed, the supernatant discarded and the pellet resuspended in 40 µL of gel loading buffer (100 mM Tris-base (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue, 200 mM DTT). The samples were boiled at 100 °C for 5 minutes and stored at -20 °C until further use.

The whole OmpK was not purified during this thesis, but some sample after a batch-based purification from a previous thesis (Fiúza Almeida Lopes, 2017) was provided. This purification method is described in the annexes.

2.3 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous polyacrylamide gel system of Laemmli (1970) was used to separate proteins according to their molecular mass. The denaturing polyacrylamide gel was prepared according following compositions (Table 2).

In the case of the whole OmpK, the 12.5 % running gel was used. For the smaller N-terminal OmpK, the running gel had a concentration of 15 % acrylamide.

Table 2: composition of denaturing polyacrylamide gel

Stock solution	Running gel [µL]		Stacking gel [µL]
	12.5 % (w/v)	**15 % (w/v)**	**4 % (w/v)**
Running buffer (1.5 M Tris base, pH 8.8)	1,875	1,875	-
Stacking buffer (0.5 M Tris base, pH 6.8)	-	-	375
Acrylamide stock (30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide)	2,080	2,500	270
H_2O	945	525	1,305
10 % (w/v) APS	50	50	30
TEMED	2.5	2.5	2.0
SDS 10 % (w/v)	50	50	20

The ammonium persulphate (APS) and the N,N,N',N'-tetramethylethylenediamine (TEMED) were added just before pouring the gel into the frame to prevent premature polymerization of the acrylamide.

When the gel was polymerized, it was immersed in running buffer 1 X (running buffer 10 X: 0.25 M Tris base, 1.92 M glycine, 1 % (w/v) SDS, pH 8.3).

The cell broth samples were prepared by centrifugation, discard of the supernatant, resuspension of the cell pellet in 40 µL gel loading buffer (100 mM Tris base, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue, 200 mM DTT) and boil for 5 minutes at 100 °C. 10 µL of the protein samples and 4 µL of a molecular mass standard (Precision Plus Protein; Dual Xtra Standards; 2 – 250 kD) were applied to each well of the gel. To separate the proteins by the molecular mass, 160 V were applied to the gel until the bromophenol blue from the loading buffer reached the end of the gel.

The polyacrylamide gels were stained by emersion in previously boiled coomassie blue staining solution (0.2 % (w/v) coomassie blue R-250, 10 % (v/v) acetic acid, 47.5 % (v/v) ethanol) for 30 minutes with gentle agitation. Afterwards, the gel was washed with distilled water and distained with previously boiled distaining solution

(10 % (v/v) acetic acid, 26.25 % (v/v) ethanol) for 20 minutes with gentle agitation. This step was carried out a second time with fresh distainer solution.

2.4 Hydrophobic interaction chromatography (HIC)

This chromatographic separation was run in an Äkta™ Purifier FPLC system from GE Healthcare (Sweden). The HIC column was a HiTrap™ Phenyl HP with 1 mL bed volume. For HIC implementation, a buffer described from Shimp et al. (2006) was used. Two almost identical buffers were prepared. The elution buffer consisted of 50 mM Tris-HCl, 1 mM EDTA, 1 M urea, pH 7.5, and the running buffer was the same as for elution but with additional 1.7 M NaCl. The salt concentration of the protein sample was adjusted to 1.7 M NaCl before injection into the column.

First, the HiTrap™ Phenyl HP column was equilibrated with 10 column volumes (CV) of running buffer at a flow of 1 mL/min. After that, 1.5 mL of the purified protein sample from Fiúza Almeida Lopes (2017) was injected and further washed with 8 CV of running buffer. The detector measured absorption of outlet liquid phase at a wavelength of 280 nm. To elute the hydrophobic components, a step change to the elution buffer was imposed and 5 CV were allowed to flow. Afterwards, pure water was pumped in the system to get rid of all the salt. For regeneration of the column, 0.5 M NaOH was used and in the end the column was stored in 0.01 M NaOH.

2.5 Methods for insertion of foreign DNA in bacterial cells

2.5.1 Preparation of competent E. coli αDH5 cells

A colony of *E. coli* αDH5 was grown overnight in 6 mL of LB liquid medium at 37 °C with 250 rpm of orbital agitation. 100 mL of LB liquid medium were inoculated with aliquots from the overnight culture to an OD_{640} of 0.1 and incubated at 37 °C and orbital agitation of 250 rpm. When the bacterial culture reached an OD_{640} of around 0.5, the cells were harvested by centrifugation in sterile centrifuge bottles at 5,600 rpm (12145 rotor, J2-21, Beckman) for 5 minute at 4 °C. For the elimination of salts, the cell pellets were washed two times. In the first washing step 100 mL of $MgCl_2$ 0.1 M was used to resuspend the pellet, following by a centrifugation at 3,400 rpm for 5 minutes at 4 °C. The second washing step was carried out with

100 mL of $CaCl_2$ 0.1 M. The bacterial cells were resuspended, placed on ice for 30 minutes and centrifuged again at 3,400 rpm for 5 minutes at 4 °C. After the last centrifugation step the pellets were resuspended in 22 mL of $CaCl_2$ 0.1 M and 3.5 mL of glycerol 86 % (w/v) was added. 500 µL aliquots of the resulting suspension were frozen in 1.5 mL cryovials and kept at -80 °C until further use.

2.5.2 Classical transformation of *E. coli* αDH5

For the classic transformation 50 µL TCM (10 mM Tris, 10 mM $MgCl_2$, 10 mM $CaCl_2$, pH 7.5) was mixed with 150 µL competent cells (*E. coli* αDH5) and 15 µL of the ligated plasmid DNA and stored for 30 minutes on ice. Next, to heat shock the cells, the samples were placed into the dry bath (Fischer Scientific) at 42 °C for 3 minutes. Then, they were again placed on ice for 5 minutes. Afterwards, 800 µL of LB liquid medium was added and the cells were incubated for 1 hour at 37 °C with 250 rpm of orbital agitation. This cell suspension was plated on appropriate selective medium agar plates (LB Tp100 or LB Ap150) and incubated at 37 °C overnight.

2.5.3 Preparation of electro-competent *E. coli* BL21 (DE3) cells

30 µL of LB liquid medium were inoculated with a colony of *E. coli* BL21 (DE3) and incubated overnight at 37 °C with 250 rpm of orbital agitation. 100 mL of LB liquid medium were inoculated with aliquots from the overnight culture to an OD_{640} of 0.1 and incubated at 37 °C with orbital agitation of 250 rpm. When the bacterial culture reached an OD_{640} of around 0.8, the cells were harvested in sterile centrifuge bottles by centrifugation at 12,000 x g (J2-21, Beckman) for 15 minutes at 4 °C. Three washing steps with sterile ice-cold distilled water were carried out to get rid of salts. Sequentially, volumes of 100 mL, 60 mL and 20 mL of the water were used for the washes and centrifuged as described before. Afterwards, the pellets were resuspended in 4 mL of 10 % (w/v) glycerol and centrifuged at 12,000 x g for 15 minutes at 4 °C. Another 2 mL of the glycerol were used to resuspend the resulting cell pellets and 110 µL aliquots were frozen in 1.5 mL cryovials and stored at -80 °C until further use.

2.5.4 Transformation of *E. coli* BL21 (DE3) by electroporation

When electro competent cells were used for the transformation, the plasmid DNA had to be diluted 1:50 first. 2 µL of this diluted solution was mixed with 110 µL of electro-competent *E. coli* BL21 (DE3) cells and transferred into an ice-cold cuvette with electrodes. This cuvette was placed into the chamber rack of a Gene Pulser™ apparatus (Bio-Rad). An electric pulse with a resistance of 4 kΩ, a capacitance of 25 µF and a voltage of 2.5 kV was applied on the cuvette and immediately afterwards 1 mL of LB liquid medium was added to the cells. The cells were incubated for 1 hour at 37 °C. This cell suspension was plated on appropriate selective medium agar plates (LB Tp100 or LB Ap150) and incubated at 37 °C overnight.

2.5.5 Plasmid DNA extraction by alkaline lysis method

Colonies were either grown in an overnight liquid culture (low copy number plasmid) or picked from the medium agar plate, both containing the respective antibiotic. From the liquid culture, 3,000 µL of the cell broth were centrifuged for 5 minutes maximum speed, the supernatant was removed, the pellet resuspended in 150 µL of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-Base, pH 8.0, 5 mg/mL lysozyme, 0.5 mg/mL RNase) and incubated for 5 minutes at room temperature. If colonies from a plate were used, cells were picked from the plate and suspended in solution I. The further protocol was the same for both types of plasmids:

- add 200 µL of solution II (0.2 M NaOH, 1 % (w/v) SDS), mix by inversion, incubate for 5 minutes on ice
- add 150 µL of solution III (3 M potassium acetate, 2 M glacial acetic acid, pH 4.5 – 5.5), mix by inversion, incubate for at least 10 minutes on ice
- centrifuge for 10 minutes at maximum speed
- remove the supernatant to a new tube
- add 1 mL cold 100 % ethanol to the supernatant, incubate for 10 minutes on ice
- centrifuge for 10 minutes at maximum speed
- remove supernatant and keep the pellet
- add 500 µL of 70 % (v/v) ethanol, vortex quickly
- centrifuge for 5 minutes at maximum speed, remove supernatant

- the DNA pellet was dried by vacuum concentration (concentrator plus, Eppendorf) at 45 °C for 15 minutes using the alcohol dry (V-AL)
- resuspend the pellet in 30 µL H_2O

2.5.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out as described by Maniatis et al. (1982). Concentrations of agarose (Seakem LE, FMC bioproducts) ranging from 0.8 % to 1 % (w/v) in TAE 1X buffer (TAE 50X – 242 g/L Tris-base, 57.1 mL/L acetic acid, 37.2 g/L $Na_2EDTA*2H_2O$) were used to separate the DNA fragments. To prepare samples prior electrophoresis, 1 µL of gel loading buffer 10X was added to each 10 µL of DNA sample solution. The molecular weight marker GeneRuler 1kb Plus DNA ladder (Thermo Scientific) was used at 0.5 µg/lane.

The extracted plasmid DNA samples were run in the agarose gel electrophoresis chamber at 100 V until the bromophenol blue line from the gel loading buffer reached about ¾ of the gel. For the staining with Green Safe, 2 µL/100 mL agarose of the nucleic acid stain was added to the agarose before pouring the gel. When the run of the gel was completed, the bands of the gel were immediately visualized with UV light in a transilluminator (Bio-Rad).

2.6 *E. coli* BL21 (DE3) Lpp antisense

2.6.1 construction of a plasmid for the induction of Lpp antisense of *E. coli* BL21 (DE3)

2.6.1.1 PCR

The oligonucleotide primers were designed based on the sequence of the gene encoding for Lpp of *E. coli* BL21 (DE3) (NCBI GenBank: CAQ32153.1) and were synthesized by STAB VIDA (Portugal) (Figure 6).

CTTGCGGTATTTAGTAGCCATGTTGTCCAGACGCTGGTTAGCACGAGCTGCGTC
ATCTTTAGCAGCCTGAACGTCGGAACGCATTGCGTTCACGTCGTTGCTCAGCTG
GTCAACTTTAGCGTTCAGAGTCTGAACGTCAGAAGACAGCTGATCGATTTTAGC
GTTGCTGGAGCAACCTGCCAGCAGAGTAGAACCCAGGATTACCGCGCCCAGTA
CCAGTTTAGTAGCTTTCAT

Figure 6: *E. coli* BL21 (DE3) Lpp antisense sequence

The primers that were used to amplify the Lpp antisense sequence are described in Table 3.

Table 3: sequence, size and melting temperature of the lower and upper primer used to amplify the *E.coli* BL21 (DE3) Lpp antisense sequence. The restriction enzyme recognition sequence is underlined. Abbreviations used: A, adenine; C, cytosine; G, guanine; T, thymine.

Primer	Sequence	Size (bp)	melting temperature (°C)
Up_EcoRI_asLPP	5'-AAG AAT TCC TTG CGG TAT TTA G-3'	22	50.8
Lw_HindIII_asLpp	5'-AAA AGC TTA TGA AAG CTA CTA AAC-3'	24	49.8

The components for the PCR mix to amplify the Lpp antisense sequence are listed in Table 4.

Table 4: reaction components used to amplify *E. coli* BL21 (DE3) Lpp antisense by PCR

DNA template (ng)	upper primer (µM)	lower primer (µM)	HF 5X buffer (µL)	dNTP's (µM)	Phusion enzyme (U)	H_2Od (µL)	total volume (µL)
5	1.0	1.0	4.0	200	0.4	required for total volume	20

DNA template was a reverse complement from *E. coli* BL21 (DE3) Braun's lipoprotein with a length of 234 nucleotides. PCR amplification was performed in a 2720 thermal cycler (Applied Biosystems) with following conditions:

- 3’ 98 °C
- 10” 98 °C
- 30” 61 °C } 30 X
- 8 “ 72 °C
- 7’ 72 °C

The PCR product was visualized on an agarose gel to check the sample purity and to see if it has the expected size of 250 bp (see 2.5.6). If so, the band was extracted using the NZY Gelpure kit (Nzytech) according to the manufacturer’s instructions. The amount of DNA purified from the gel was quantified by measuring the A_{260} in a ND-1000 Spectrophotometer (NanoDrop).

2.6.1.2 Restriction with EcoRI and HindIII

Around 500 ng of the from the agarose extracted *E. coli* BL21 (DE3) Lpp antisense insert DNA were mixed with 2 µL Buffer R 10 X, 10 U EcoRI, 10 U HindIII and the amount of water to get a total volume of 20 µL. For preparation of the plasmid, around 500 ng of pMLBAD were mixed with 2 µL Buffer R 10 X, 10 U EcoRI, 10 U HindIII and the amount of H_2O to get the total volume of 20 µL. This mix was prepared three times and the insert four times to get the right amount of restricted DNA. The restrictions were carried out at 37 °C overnight. The restriction enzymes used are from Thermo Scientific.

2.6.1.3 Ethanol precipitation of DNA

The restricted samples (section 2.6.1.2) were ethanol precipitated to concentrate and desalt the DNA samples. The samples of the same type were combined and sodium acetate (3 M) was added in the volume ratio of 1:10 (solution:sample) and cold 100 % ethanol 2.5 times of the sample volume. This mix was stored at –80 °C for at least one hour up to overnight. After that, the samples were centrifuged for 30 minutes with 17,600 x g (Sigma 2K15) at 4 °C. The resulting supernatant was discarded, 300 µL of 70 % (v/v) ethanol were added to wash the pellet and centrifuged for more 15 minutes. Again, the supernatant was discarded and the pellet was dried on speed vacuum (Concentrator plus, Eppendorf) for 15 minutes at 45 °C

with the alcohol dry (V-AL) program. The dry pellet was resuspended in 20 µL H_2O DNA grade.

2.6.1.4 DNA ligation

To calculate the needed amounts of insert and plasmid, the concentration of DNA was measured using a ND-1000 spectrophotometer (NanoDrop). 50 ng of EcoRI/HindIII restricted pMLBAD plasmid was mixed with the EcoRI/HindIII restricted insert Lpp antisense for a molar ratio of 1:5. Additionally, 2 µL T4 DNA ligase buffer 10 X, 2.5 U T4 DNA ligase (Thermo Scientific), and the amount of H_2O to get a total volume of 20 µL were added. The samples were incubated for 1 hour at 22 °C. The ligation reaction was heat-inactivated at 70 °C for 5 minutes. The ligated DNA samples were stored at -20 °C until further use.

After the ligation, the DNA was transformed into *E. coli* αDH5 cells (see section 2.5.2).

2.6.1.5 Confirmation of transformants by restriction enzyme digestion and sequencing

To see, if the *E. coli* transformants host the correct plasmid construct, the plasmid from each colony transformant was extracted by alkaline lysis method (section 2.5.5) and was cut with the restriction enzyme SalI. A negative transformant (pMLBAD without insert) cut with SalI showed two bands with the sizes of 4,647 and 2,128 nucleotides and a positive transformant (pMLBAD plus Lpp antisense insert) would show just one band with 6,959 nucleotides.

The mix for the restriction enzyme digestion included 10 U of SalI (Thermo Scientific), 3 µL of the buffer, about 1,000 ng of extracted plasmid DNA and the amount of H_2O to get a total volume of 30 µL. It was incubated for at least 3 hours at 37 °C. To separate the DNA samples by agarose gel electrophoresis, the loading buffer 10 X (0.25 % (w/v) bromophenol blue (Merck), 0.25 % (w/v) xylene cyanol FF (Sigma), 30 % (v/v) glycerol) was added with the ratio 1:10 to the total volume of the restricted samples. How the agarose gel electrophoresis was carried out can be seen in 2.5.6)

If a correct transformant was found, the plasmid DNA was extracted using the NZY Miniprep Kit (Nzytech) and transformed into *E. coli* BL21 (DE3) cells. For a second

confirmation, the extracted plasmid was also sent for sequencing by the company Eurofins Genomics (Germany) using the primer pBAD_RWD. The obtained sequence was aligned with the expected DNA sequence of the plasmid construct using the blastn tool resident at the National Center for Biotechnology Information (NCBI).

2.6.2 Physiological tests

2.6.2.1 E. coli growth curves

The OD_{640} was measured during the growth of *E. coli* BL21 (DE3), BL21 (DE3) with pMLBAD and BL21 (DE3) with pAR2 to see, if the plasmid and the induction of the antisense Lpp have an effect on the growth. To do this, preinoculums were prepared in 20 mL of LB liquid medium, incubated overnight at 37 °C with 250 rpm of orbital agitation. For the cells that contain pAR2, 100 ng/mL trimethoprim were added. From these preinoculums, 50 mL of LB liquid medium with 0.5 % (w/v) arabinose were inoculated to a starting OD_{640} of 0.05. The first sample was taken after one hour. To follow the exponential phase, following samples were taken every 30 minutes until the OD_{640} indicated that stationary phase was reached (T5.5). Afterwards, two more samples (T6.5 and T7.5) were taken.

2.6.2.2 Ethidium bromide (EtBr) influx assay

To test, if the induction of the antisense Lpp transcript has an effect on the outer membrane permeability, an ethidium bromide influx assay was performed. Plates based on LB medium with different amounts of ethidium bromide (0 mg/L, 1 mg/L, 2 mg/L) and 0.5 % (w/v) of L-arabinose to induce the promoter were prepared. To see, if the plasmid is lost during this test, plates with and without 350 µg/mL of trimethoprim were used. As the intercalating agent is photosensitive, the plates were covered in aluminium foil and stored at 4 °C until use.

The three types of bacteria were tested on the ethidium bromide plates: *E. coli* BL21 (DE3), BL21 (DE3) containing pMLBAD and BL21 (DE3) containing pAR2. These bacteria were streaked on the plates and incubated for 24 hours at 37 °C, again protected from light through aluminium foil. To detect the fluorescence in the cell colonies, UV light from a transilluminator (Bio-Rad) was used. After the visualisation, the plates were put to 4 °C and visualised again after further 24 hours.

2.6.2.3 Propidium iodide staining

The samples for this test were taken from a growth of *E. coli* BL21 (DE3) containing the pMLBAD vector and another growth of BL21 (DE3) pAR2. Each, 5 mL of LB medium plus 150 ng/mL trimethoprim were inoculated with the cells and grown at 37 °C with 250 rpm of orbital agitation overnight. With this preinoculums, each 20 mL of LB medium (10 g/L NaCl) plus trimethoprim (150 ng/mL) were inoculated to an OD_{640} of 0.1 and samples were taken after 1.5, 2.5 and 4.5 hours. These samples were centrifuged at 13,500 rpm for 3 minutes to remove the medium and the pellet was resuspended in PBS 1X (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4) to wash the cells. After another centrifugation step, the pellets were resuspended in 200 µL propidium iodide solution (75 µmol/L) and incubated for 10 minutes, followed by another washing step. The resulting pellet was resuspended again in 200 µL of PBS 1X and afterwards put in a 96-well-plate to measure the fluorescence (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). For excitation, a wavelength of 530 nm was used and for emission 590 nm.

2.6.2.4 Susceptibility to salts

E. coli BL21 (DE3), BL21 (DE3) + pMLBAD and BL21 (DE3) + pAR2 were grown each in LB liquid medium, supplemented with 100 µg/mL trimethoprim when required. The three cell cultures were grown at 37 °C with orbital agitation of 250 rpm for 3 hours. Afterwards, cells were harvested by centrifugation at 13,500 rpm for 3 minutes and resuspended with sterile NaCl 0.9 % (w/v) to an OD_{640} of 1.0. Serial dilutions were prepared up to 10^{-7}. 10 µL of each dilution was spotted on the agar plate. The plates that were used here contained LB agar medium with additional 2.5 % (w/v) NaCl and 0.5 % (w/v) arabinose for the induction. The spots were allowed to dry and the plates were incubated at 37 °C for 24 hours. Colonies were counted thereafter for osmotolerance analysis.

2.6.2.5 Susceptibility to SDS

For this test cells were grown and prepared as at the test for susceptibility to salts (2.6.2.4). The LB agar plates were supplemented with 0.5 % (w/v) SDS, 1 mM EDTA

and 0.5 % (w/v) arabinose. After incubation of the plates, the cells were counted to analyse their resistance to the detergent.

2.7 Truncated form of OmpK 1-99 from *Vibrio alginolyticus* ATCC 17749

2.7.1 Construction of a plasmid for overproduction of the truncated form of OmpK 1-99

2.7.1.1 PCR

The oligonucleotide primers were designed based on the sequence of the gene encoding for OmpK of *Vibrio alginolyticus* ATCC 17749 (GenBank: AGV17311.1) and were synthesized by STAB VIDA (Portugal) (Table 5).

The *ompK* nucleotide sequence had been previously cloned in pJBF2 plasmid. Therefore pJBF2 plasmid was used as DNA template for amplification of the nucleotide region encoding the truncated form of OmpK 1-99. This plasmid was extracted using the NZY Miniprep Kit (Nzytech). The PCR components were used in the same concentrations as in the PCR of section 2.6.1.1 (see Table 4) but the primers described in Table 5 were used instead.

Here, the primers described in Table 5 were used.

Table 5: Sequence, size and melting temperature of the lower and upper primer to amplify the truncated form of OmpK 1-99 from *V. alginolyticus* ATCC 17749. The restriction enzyme recognition sequence is underlined. Abbreviations used: A, adenine; C, cytosine; G, guanine; T, thymine.

Primer	Sequence	Size (bp)	melting temperature (°C)
Up_OmpK_P1-99	5'-AAC TCG AGC ATA CGT GGA GC-3'	20	48.7
LW_OmpK_P1-99	5-AAC ATA TGC GTA AAT CAC TTT TA-3	23	57.2

The DNA template was pJBF2 from Fiúza Almeida Lopes (2017).

PCR amplification was performed in a 2720 thermal cycler (Applied Biosystems) with following conditions:

- 2’ 98 °C
- 10” 98 °C
- 30” 58 °C } 30 X
- 10 “ 72 °C
- 7’ 72 °C

A 1 % agarose gel was used to see, if the PCR product has the expected size of 316 nucleotides and if it is pure. If so, the bands were cut out and purified with the NZY Gelpure kit (Nzytech). The purification was carried out according to the manufacturer’s instructions. The concentration of DNA purified from the gel was checked by measuring the A_{260} in a ND-1000 Spectrophotometer (NanoDrop).

2.7.1.2 Restriction

The purified PCR fragment, as well as the pET23a+ plasmid, was restricted with two different enzymes, XhoI and NdeI. About 500 ng of DNA were mixed with 2.5 µL buffer O 10 X, 2 µL XhoI, 1 µL NdeI and as much H_2O to have a total volume of 25 µL. The restriction was carried out at 37 °C for 3 hours.

Basically, the further steps (precipitation, ligation, transformation and minipreps) were carried out as described above (2.6.1.3, 2.6.1.4, 2.5.2, 2.5.5). Because pET23a+ was used as vector, LB plates supplemented with ampicillin were used for the selection after the transformation.

2.7.1.3 Confirmation of transformants by restriction enzyme digestion and sequencing

The extracted plasmids from each colony transformant were restricted with BglII. Through the use of this enzyme a negative construct (pET23a+ without insert) results in just one band with the size of 3,666 nucleotides and a positive construct (pET23a+ plus truncated OmpK 1-99 sequence) shows two bands, one with 3,515 and a second band with 374 nucleotides.

For the restriction enzyme digestion, 10 U of BglII (Thermo Scientific), 3 µL of the buffer, about 1,000 ng of extracted plasmid DNA and the amount of H_2O to get a total volume of 30 µL were mixed. To separate the DNA samples by agarose gel electrophoresis, the loading buffer 10X was added with the ratio 1:10 to the total

volume of the restricted samples. To visualize the product of the restriction, 1 % (w/v) agarose gels were used (described above in section 2.5.6).

If the gel showed a correct transformant, the plasmid DNA was extracted using the NZY Miniprep Kit (Nzytech) and transformed into *E. coli* BL21 (DE3) cells. Also, the extracted plasmid was sent for sequencing by the company Eurofins Genomics (Germany). The blastn tool resident at NCBI was used to align the obtained sequence with the expected DNA sequence of the plasmid construct.

2.7.2 Optimization of the truncated OmpK 1-99 overexpression

2.7.2.1 Overexpression at 37 °C

The first kind of overexpression was carried out according to the vectors manual (Novagen, 2018b). A preinoculum was prepared using 50 mL LB liquid medium containing ampicillin (150 µg/mL) and the *E. coli* BL21 (DE) cells containing the pAR1. The cell broth was incubated at 37 °C and with orbital agitation of 250 rpm until it reached an OD_{640} of around 1. 100 mL of SB liquid medium supplemented with 150 µg/mL of ampicillin was inoculated to an initial OD_{640} of 0.1 with the preinoculum and incubated at 30 °C with orbital agitation of 250 rpm. When the culture reached an OD_{640} of around 0.6, the T7 promoter was induced with 0.4 mM of IPTG. After that, the cultures were incubated further under the same conditions. Samples were taken after every hour (until T4) and overnight (T20). Overexpression was checked by carrying out an SDS-PAGE (section 2.3) with the protein extracts obtained from sampled *E. coli* cultures.

2.7.2.2 Overexpression at 37 °C with induction at high OD_{640}

Malik et al. (2016) prove that induction of the plasmid at a high cell density can result in higher OD_{640} 3 hours after induction, compared to induction at OD_{640} at 0.6.

Therefore, one attempt to facilitate the production of N-terminal OmpK was to induce at a higher OD than usual. In this case, more cells are available to produce the protein. For the preinoculation *E. coli* BL21 (DE3) with pAR1 was grown in 20 mL of LB liquid medium with 150 µg/mL ampicillin for about 2 hours. An aliquot of the preinoculum was used to inoculate 50 mL of SB liquid medium to get an OD_{640} of 0.1. The cells were grown further at 37 °C with 250 rpm orbital agitation. When the cell

culture reached an OD_{640} of 1.9, 0.4 mM IPTG was added and growth was continued at the same conditions. To minimize the time in which the cells can degrade the protein, this overproduction was stopped after 2 hours. Samples from the cell broth were taken before the induction, 0.5, 1 and 2 hours after induction to check the cells' protein production.

2.7.2.3 Overexpression at 16 °C

To improve the yield of soluble protein, the pET23a+ manual (Novagen, 2018b) proposes prolonged induction at low. The preinoculum and inoculation were prepared as in section 2.7.2.1. After inoculation, the cells were grown at 30 °C with 250 rpm orbital agitation until the cells reached an OD_{640} of 0.6. At this cell density they were induced with 0.4 mM IPTG and grown overnight at 16 °C and with orbital agitation of 250 rpm. Cell cultures were sampled for SDS-PAGE analysis just before and 16 hours after induction.

2.7.2.4 Overexpression by autoinduction

For *lac* operon controlled expression systems, autoinduction is a way of recombinant protein production without the need to add an inducer. A mixture of different carbon substrates (glucose, glycerol and lactose) is used, where the different components are used sequentially according to the preference of the organism. First, glucose is used up, followed by glycerol and lactose as the last. The latter one induces the *lac* operon and therefore the controlled protein production. In that way, the change from growth to recombinant protein production is carried out by the expression host itself (Li et al., 2011).

The preinoculum was prepared as described in section 2.7.2.1. The medium for the autoinduction consisted of 48 mL ZY (10 g/L tryptone, 5 g/L yeast extract), 1 mL 50 X M (177.5 g/L $NaHPO_4$, 170.0 g/L KH_2PO_4, 134.0 g/L NH_4Cl, 35.5 g/L Na_2SO_4), 1 mL 50 X 5052 (250 g/L glycerol, 25 g/L glucose, 100 g/L α-lactose monohydrate) and 100 µL $MgSO_4$ (1 M). It was inoculated to a start OD_{640} of 0.05 and incubated at 37 °C with orbital agitation of 250 rpm. Samples were taken after 2, 4 and 18 hours after inoculation.

2.7.3 Purification by immobilized metal affinity chromatography (IMAC)

For the purification of the truncated form of OmpK 1-99 protein the cells from the overexpression were harvested through centrifugation (5 minutes, 7,000 x g, 4 °C, J2-21 Beckman) and the pellet was resuspended in sodium phosphate buffer 1X (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 20 mM imidazole. The cells in this suspension were then disrupted by sonication. With a Branson Sonifier 250 (Branson) the cells were disrupted with eight cycles of 30 seconds, 50 % of duty cycle, output of 60 and two minutes beak between each cycle. The cell debris was sedimented by centrifugation (aliquoted for 60 minutes, 12,000 rpm, 4 °C, J2-21 Beckman) and the soluble protein fraction (supernatant) was kept at 4 °C until application on an IMAC column (HisTrap™ FF 1 mL, GE Healthcare). GE Healthcare Bio-Sciences (2018) provides a manual for the use of the column. After application of 10 CVs of sodium phosphate buffer with 20 mM imidazole on the IMAC column (equilibration), the supernatant containing the recombinant peptide was passed on the column (washing). Afterwards, further 10 CVs of the sodium phosphate buffer with 20 mM imidazole were passed on the column. To elute the bound protein from the IMAC column, the concentration of imidazole was consecutively increased by passing sodium phosphate buffer with 60 mM, 100 mM, 250 mM and 500 mM imidazole, 5 CVs each, on the column. The eluted liquid was collected in aliquots of 1 mL. In the end, 10 CVs of sodium phosphate buffer with 20 mM imidazole was passed on the column and it was closed and stored at 4 °C. The second millilitre of each elution buffer with different imidazole concentration was used to check the proteins through SDS-PAGE and Western-Blot.

2.7.4 Western-Blot

For gels that were used for Western-Blot, a positive control (BCAL2645 from *B. cenocepacia* J2315, overexpressed from construct pSAS36 (pET23a+ with BCAL2645 gene)) was additionally applied. After SDS-PAGE, the polyacrylamide gel was immersed in 30 mL transfer (Bjerrum and Schafer-Nielsen) buffer (48 mM Tris base, 39 mM glycine, 20 % (v/v) methanol, 0.04 % (w/v) SDS pH 9.2) for at least 15 minutes. The nitrocellulose (NC) membrane (PALL Corporation) and the 3MM Whatmann filter paper to be used were immersed in the transfer buffer for 5 minutes. With these components the system was assembled as shown in Figure 7.

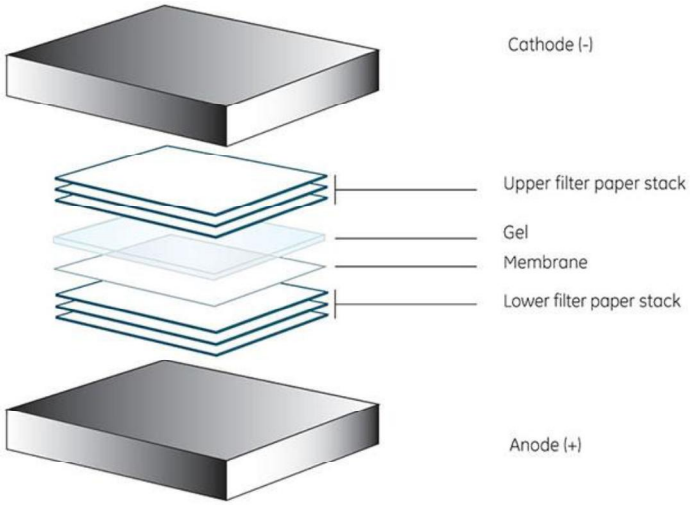

Figure 7: construction of a gel-membrane sandwich in the semidry transfer unit (GE Healthcare Life Sciences, 2018)

The electro 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, Bio-Rad). After the transfer of the proteins from the gel to the membrane, the membrane was blocked by incubation with 30 mL of blocking buffer (5 % (w/v) skim milk in PBS 1 X) with gentle agitation at 4 °C overnight or for 1 hour at room temperature. Next, the membrane was washed three times for 5 minutes each with 20 mL of washing buffer (0.05 % (v/v) Tween 20 in PBS 1X). Then the membrane was incubated with 20 mL of antibody solution (washing buffer containing 1.5 % (w/v) BSA and 1:2000 dilution of monoclonal anti-polyhistidine peroxidase conjugate clone His-1 antibody (SIGMA)) for 90 minutes at room temperature with gentle agitation. Afterwards, the membrane was washed again three times as before. The next step was to add 2 mL of enhanced chemiluminescence (ECL) detection reagent (solution 1: 2.5 mM luminol, 400 µM p-coumaric acid, 100 mM Tris-HCl, pH 8.6; solution 2: 0.15 % (v/v) H_2O_2, 100 mM Tris-HCl pH 8.6, mixed in the ratio 1:1) and the membrane was incubated for 5 minutes with gentle agitation. The excess of reagent was removed and the detection was performed using the chemiluminescence imaging system Fusion Solo (Vilber Lourmat).

3. Results and Discussion

3.1 HIC and overexpression of the whole OmpK

The OmpK protein sample used originates from Fiúza Almeida Lopes (2017), who denaturated and renaturated the protein from inclusion bodies and purified it. Afterwards the OmpK protein solution was passed through a column to remove endotoxins, but this was not successful. Therefore, it was tried to separate the endotoxins from the protein through HIC.

The resulting diagram of the values from the HIC is shown in Figure 8. The different lines correspond to the main parameters: detector signal (red), conductivity (green) and rate of elution buffer that was used.

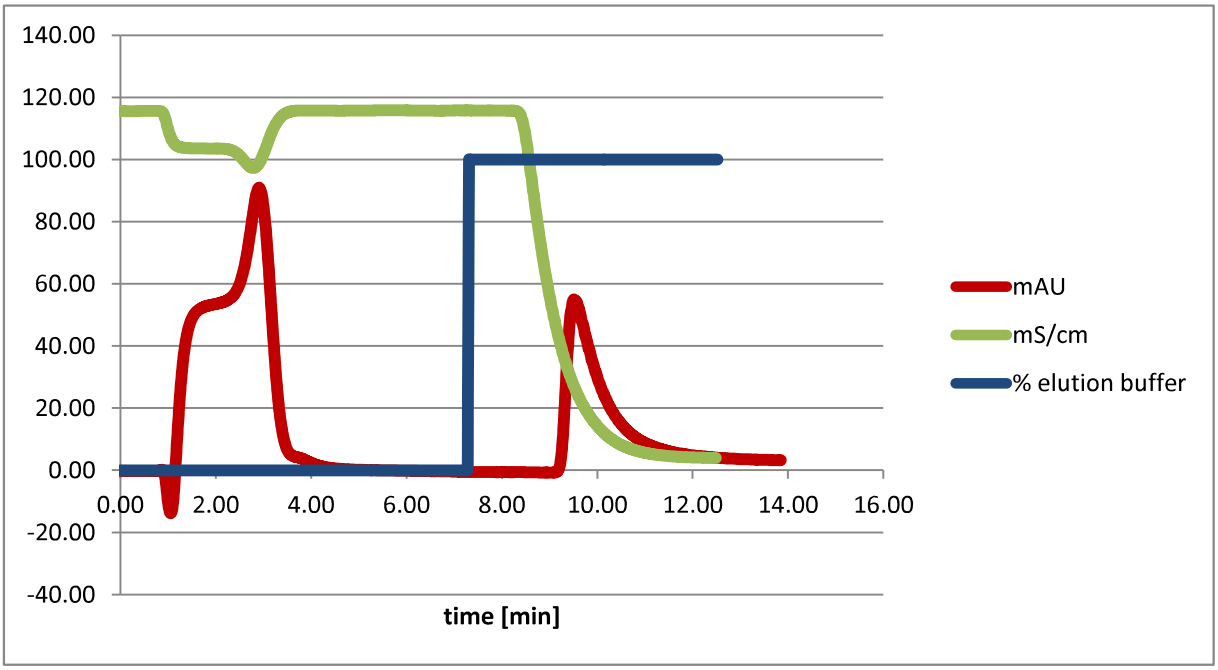

Figure 8: performance of HIC injected with purified OmpK protein solution; the red line corresponds to the signal which was obtained by detection at a wavelength of 280 nm; the green line shows the electrical conductivity; the blue line represents the profile of percentual elution buffer

The small drop of conductivity in the beginning was caused by the injection of the OmpK protein sample, which can be seen as the profile looks identical to the first peak. Even when it was tried to add the amount of salt to the protein solution to get an equal concentration as in the buffer, this drop shows that it was not achieved

completely. But as this drop is quite small, the difference in salt concentration was not significant.

The signal that is shown by the red line was obtained by an UV detector measuring at 280 nm. The first peak should in theory correspond to the OmpK protein. The weird shape of the peak (with a shoulder) could mean that the protein is not present in just one form but maybe their molecules show various confirmations or some degradation (several months of frozen storage) and so the hydrophobicity varies as well. When the first peak clearly exited the column, the buffer was changed to that one without the salt. More or less immediately after the change a second peak appeared. Theoretically, this one should correspond to the endotoxins which are much more hydrophobic and, in the presence of the high NaCl concentration interact strongly with the phenyl ligands of the stationary phase.

To analyse the peaks, fractions of them were taken: start, middle and end of the first peak and first and second half of the second peak. These samples were concentrated and prepared to use for an SDS-PAGE. Additionally, a sample of the initial protein solution, which was injected in the HIC column, was applied on the gel. The resulting gel can be seen in Figure 9.

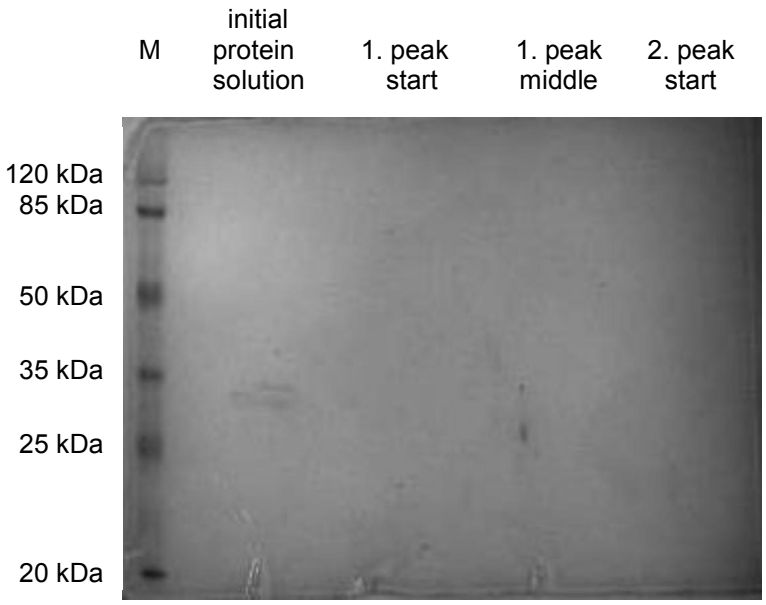

Figure 9: stained SDS-PAGE gel with protein fractions eluted from the HIC; Lane 1: protein standard, Lane 2: injected protein solution, Lane 3: sample from the first part of the first peak, Lane 4: sample from the middle part of the first peak, Lane 5: sample from the first part of the second peak

The only bands that are visible belong to the initial protein solution. The fractions from the two peaks from the HIC seem to be too diluted with the buffer that there was not enough protein left to be seen on the gel. The bands that can be seen from the initial protein solution are in the correct size of OmpK (31.8 kDa), but even these bands are not as strong as expected. As the purified protein was stored at -80 °C for several months, it is possible that its molecules degraded somehow and so there is less native OmpK protein left in the liquid. This could be also the reason for the second band. The lower band is wider than the one above. This can be due to the fact that 30 µL of each fraction was loaded on the gel and so the well maybe got widened by the huge amount of sample. The higher amount of HIC fractions was used to increase the probability of visualization of thin bands; it was known that the concentration of the protein would be really small.

To obtain a bigger amount of OmpK protein, the whole OmpK was overexpressed.

The Coomassie Blue stained SDS-PAGE gel with the protein samples from the overexpression of the whole OmpK can be seen in Figure 10. In the first lane, the molecular mass standard (Precision Plus Protein; Dual Xtra Standards; Catalog # 161-0377) was applied. The further lanes contain the protein samples of 3 different overexpression batches, one with 100 mL of SB culture volume and two with 200 mL each. T0 corresponds to the sample that was taken before induction with IPTG and T16 16 hours after induction.

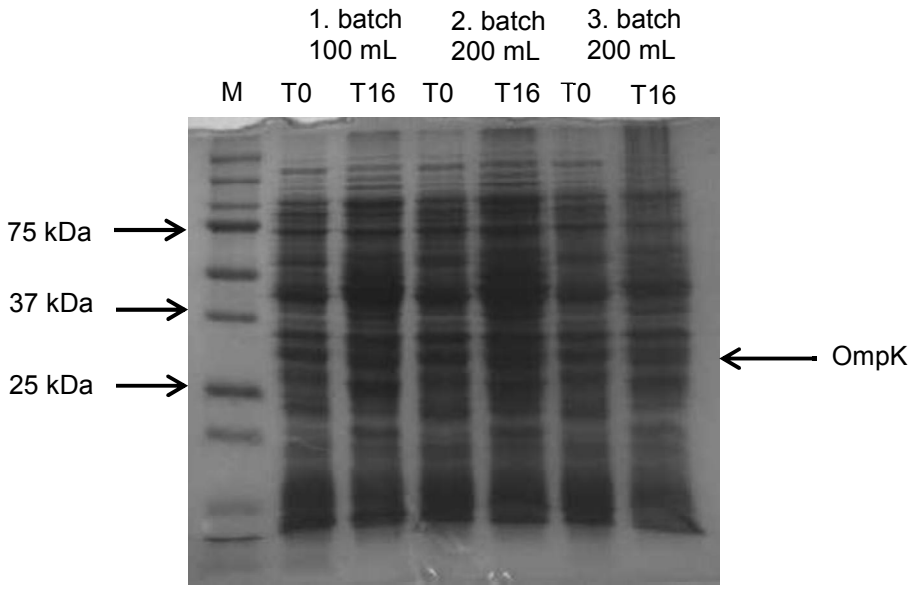

Figure 10: stained SDS-PAGE gel with protein samples from the *E. coli* overproduction of the whole OmpK; Lane 1: protein standard, Lanes 2 & 3: samples from the first overexpression (100 mL media volume) 0 and 16 hours after induction, Lanes 4 & 5: second overexpression (200 mL media volume) 0 and 16 hours after induction, Lanes 6 & 7: third overexpression (200 mL media volume) 0 and 16 hours after induction

The size of the his-tag recombinant protein was predicted with the ProtoParam tool available at ExPAS using the nucleotide sequence that encodes the OmpK protein from *V. alginolyticus* ATCC 17749. With a size of 31.8 kDa, an increase of one of the bands between the 37 kDa and 25 kDa protein standard bands is expected. On a closer look, a small increase of the concentration of OmpK can be seen in the T16 samples. This indicates that the plasmid is induced by the addition of IPTG and the protein is overexpressed. Unfortunately, the increase is pretty small. Attempts to achieve a higher overproduction could be tried looking at different culture media, temperatures for growth or also the concentration of IPTG that is used for induction.

Besides the work with the whole OmpK, it was tried to overexpress an OmpK fragment (truncated OmpK 1-99), which should be easier to express and purify than the whole protein, and it was worked on an Lpp antisense construct.

3.2 Strategy for *E. coli* BL21 (DE3) outer membrane permeabilization using Lpp antisense technology

3.2.1 Construction of a plasmid for the induction of Lpp mRNA antisense of *E. coli* BL21 (DE3)

The work on the *E. coli* Lpp antisense construct was carried out to be able to permeabilize the cell envelope of the expression host by co-expression. In that way

the overexpressed OmpK protein can be transferred from the periplasm to the cytoplasm and so the purification of OmpK is easier.

The agarose gel which was used to visualize the PCR products with the Lpp antisense sequence can be seen in Figure 11. As the two bands are slightly below the, unfortunately difficult to see, 300 bp DNA ladder band, the size of the fragment of 250 bp can be confirmed.

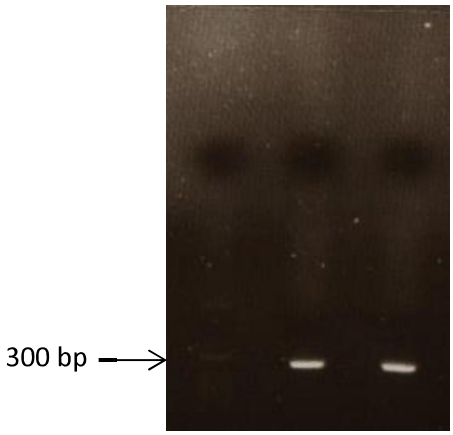

Figure 11: agarose gel; lane 1 - GeneRuler™ Low Range DNA Ladder; further lanes - PCR products from two PCR batches amplifying *E. coli* Lpp antisense sequence

The sequence of the recombinant plasmid pAR2 was obtained with Serial Cloner 1.3. This sequence and the one from pMLBAD without insert were used to draw the vectors with NEBcutter V2.0 (Vincze et al., 2003) (Figure 12). The list of ORFs does not contains Open Reading Frames only, but general plasmid features.

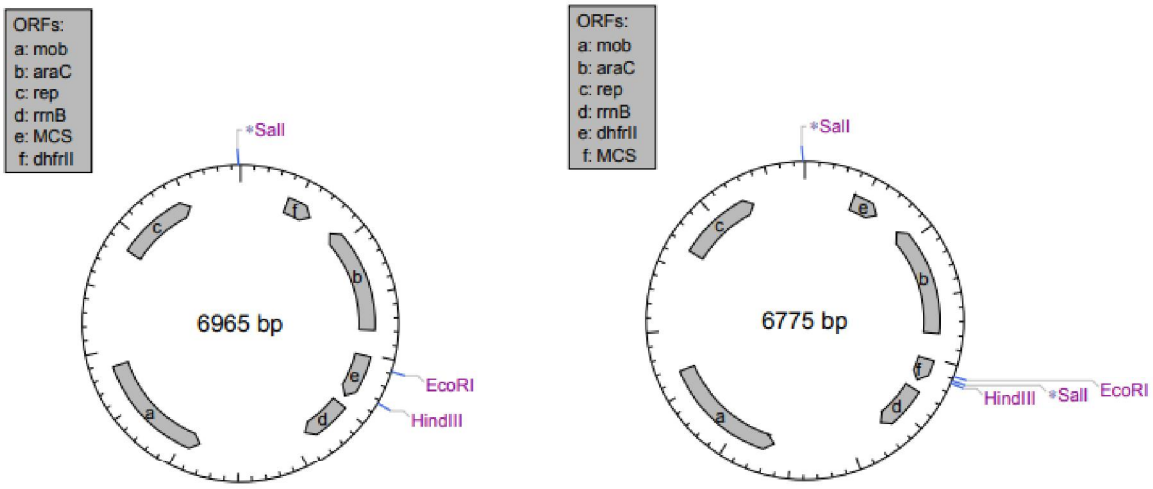

Figure 12: left - pAR2 vector (pMLBAD + Lpp antisense). right – pMLBAD without insert; restriction sites of used enzymes (EcoRI, HindIII, SalI) and plasmid features are indicated (Vincze et al., 2003)

In the pAR2 vector the Lpp antisense sequence is located between the restriction sites for EcoRI and HindIII. When this region is compared between the two vectors, it can be seen that pMLBAD has an additional restriction site for SalI there. This is the reason why this enzyme was used to distinguish between pAR2 and pMLBAD.

Following Figure 13 shows the agarose gel with the confirmation of the pMLBAD vector containing the correct Lpp antisense sequence. To see the size of the fragments, two markers were used as comparison. Lane 1 shows the GeneRuler™ 1 kb DNA Ladder and lane 4 the GeneRuler™ Low Range DNA Ladder.

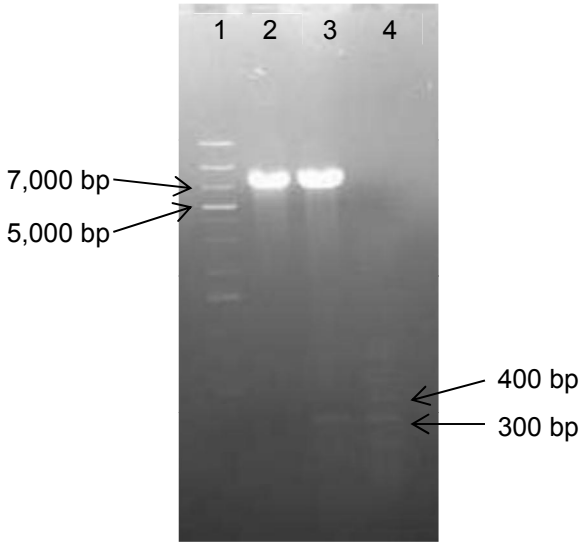

Figure 13: confirmation pMLBAD + Lpp antisense gene sequence. Lane 1: 1 kb protein ladder. Lane 2: construct cut with SalI. Lane 3: construct cut with EcoRI and HindIII. Lane 4: LR protein ladder

Lanes 2 and 3 contain the pMLBAD vector including a sequence with the correct size of 250 nucleotides, but cut with different enzymes. In lane 2 the construct was cut with SalI, which results in just one band with the size of 6,959 nucleotides. This enzyme was used to screen the transformants and to see if the plasmid was ligated with the insert. If the plasmid would have recircularized, and so has not included an insert, two bands were seen with the sizes of 4,647 and 2,128 nuceotides, respectively. The reason for this is that the part of the plasmid which was eliminated through the first restriction with EcoRI and HindIII, to obtain the sticky ends for the ligation, contained a restriction sequence for SalI. This means that if the vector recircularized, the enzyme can cut a second time, but if the insert is present, there is just one restriction site for that enzyme in the vector backbone.

In lane 3 the construct was cut with the enzymes from the first restriction, EcoRI and HindIII. When the construct is cut with these enzymes, it results in 2 bands, a big one corresponding to the empty vector (6,775 nt) and one for the insert (250 nt).

As the concentration of the plasmid solution was very high, the bands are very thick and therefore it is pretty hard to identify the exact size. But as the bands are in the correct place and a small difference in their heigh can be seen it is assumed that the construct is correct.

3.2.2 Physiological tests

3.2.2.1 E. coli growth curves

The reason to carry out the growth curves was to see, if the growth of cells containing the plasmid and plasmid with the Lpp antisense sequence looks different from cell without plasmid.

The measured OD_{640} values of the growing cultures of original and transformed *E. coli* BL21 (DE3) cells were plotted and can be seen in Figure 14. The growth was carried out in triplicates and the average of these values was used for the graph.

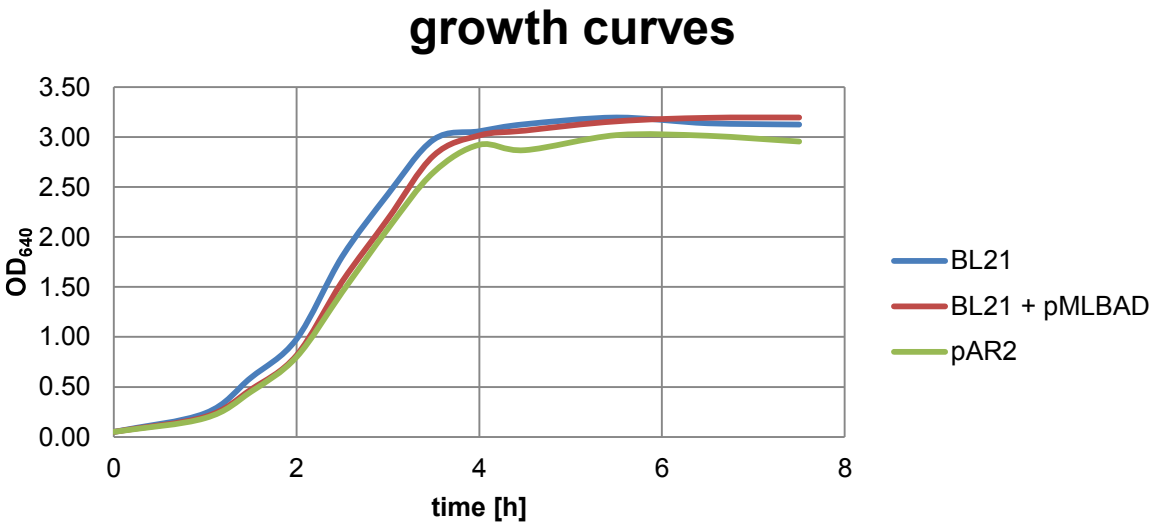

Figure 14: growth curves of *E. coli* BL21 (DE3) without plasmid, BL21 (DE3) with pMLBAD vector and pAR2 grown in LB medium at 37 °C with 250 rpm orbital agitation

The cultures were carried out and growth curves plotted to see if the plasmid and the insert have an effect on the growth of the cells. On the diagram one can observe that the fastest growing cells are the BL21 (DE3) without any plasmid inside. In general, the BL21 (DE3) containing pMLBAD grew slower than the one without the plasmid. The cells with the slowest growth were the ones containing pAR2. This indicates that the cells need to put some strength in maintaining the plasmid and even more if the insert is expressed. Therefore, cells that contain a plasmid grow slower than those that do not contain one. As the observed differences among growth curves are pretty small, this test should be repeated to see if this trend was just coincidence or can be seen systematically.

3.2.2.2 Ethidium bromide influx assay

This assay was carried out to see, if through the induction of the Lpp antisense gene the outer membrane of the *E. coli* cells is leaky and therefore more of the dye can enter the cells.

Pictures of the ethidium bromide plates can be seen in Figure 15. Not all of the used plates are shown here, just the ones without the antibiotic, because the visual evaluation is the easiest with them.

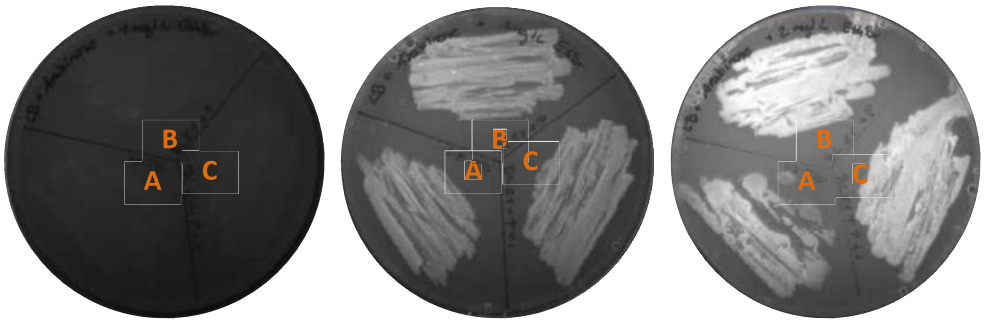

Figure 15: LB agar plates containing 0.5 % arabinose for induction and 0 mg/L (left), 1 mg/L (middle) and 2 mg/L ethidium bromide; no antibiotic in these plates; A – *E. coli* BL21 (DE3), B – BL21 (DE3) + pMLBAD, C – pAR2; incubation of plates: 24 hours, 37 °C; pictures taken under UV light

As expected, the plates without ethidium bromide showed no fluorescence at all. When nothing of the ethidium bromide is present, no component of the plates or the cells is fluorescent. The plates with 1 mg/L ethidium bromide show some

fluorescence and the ones with the double amount of ethidium bromide show even more. It is difficult to see a difference between the different cells on each plate. On the plates with 1 mg/L ethidium bromide all of them show the same intensity of fluorescence. The BL21 cells (control) on the plates with 2 mg/L ethidium bromide show a lower fluorescence than the others. In this case it seems likely that the difference is due to lower density of the cells on the plate. Maybe less cells were inoculated on the plate and therefore the fluorescence signal is lower. The plates that contained the antibiotic (results from an experiment parallel to that in Figure 15). looked similar to the ones without it, with the exception that on the portion of the plate surface in which the BL21 (DE3) cells without plasmid had been stretched the cellular growth was inhibited and therefore no cells were growing there.

3.2.2.3 Propidium iodide test

Identical to the ethidium bromide influx assay (section 3.2.2.2), also with the propidium iodide test was used to see, if *E. coli* cells get leakier through induction of the Lpp antisense sequence.

The values obtained for the fluorescence measurements of the propidium iodide test are listed in Table 6.

Table 6: fluorescence intensity results from propidium iodide test measured with a plate reader with the wavelengths for excitation at 530 nm and emission at 590 nm after staining and washing steps

	BL21 + pMLBAD [rfu]	**BL21 + pAR2 [rfu]**
beginning exponential phase	32,030	28,573
middle exponential phase	40,625	22,576
end exponential phase	29,843	28,614

The different cell type do not show a big difference. Basically, the cells that contain pMLBAD without the insert should not show any difference in fluorescence as the permeability of the cells should not vary between the different phases of growth. The values in Table 6 show small differences and are not significant. The cells containing pAR2 should take up more of the propidium iodide in the cell suspensions of middle

and end of exponential phase, as they were induced at an OD_{640} of 0.6 because the Lpp expression should have been impaired (at least partially) and the cell envelope should have become permeable to the fluorescent dye. The value of the cell suspension from the middle exponential phase is even lower than the previous one. The late exponential phase shows a second increase to approximately the value from the beginning. In general, the difference is not big enough to be significant.

In total it can be said that this experiment does not show the expected values. This test should be repeated and carried out in at least triplicates.

3.2.2.4 Susceptibility to salts

With this test it was tried to see if through a permeable cell envelope caused by the induced Lpp antisense sequence more salt is taken up from the cells. If that is the case, less cells are viable because a too high salt concentration in the cells is toxic to them.

One example for the LB agar plates with the higher amount of salts to test the susceptibility of E. coli strains to high medium osmolarity is shown in Figure 16.

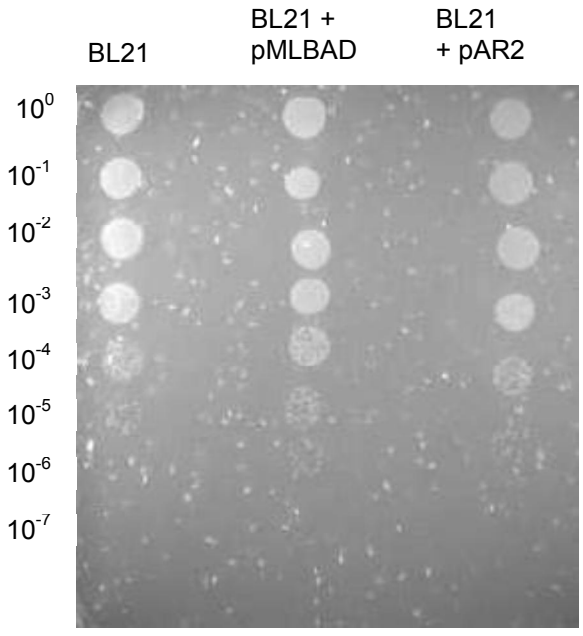

Figure 16: LB agar plate with additional 2.5 % (w/v) NaCl with cell suspension dots from *E. coli* BL21 (DE3), BL21 (DE3) with pMLBAD and BL21 (DE3) with pAR2; the initial cell suspension (10^0) had an OD of 1 and was used for serial dilutions up to 10^{-7}

If the dots of the different cells from the same dilution are compared, it looks like the middle ones (BL21 + pMLBAD) grew more than the others. To have a better impression, the colonies on three plates were counted and the average values are depicted in Table 7.

Table 7: *E. coli* cell counts (in CFU/10 µL) from the test of cells susceptibility to salt

dilution	BL21	BL21 + pMLBAD	BL21 + pAR2
10^{-4}	2.34×10^{6}	2.63×10^{6}	2.72×10^{6}
10^{-5}	3.30×10^{6}	5.00×10^{6}	4.20×10^{6}
10^{-6}	2.00×10^{6}	7.00×10^{6}	6.00×10^{6}

E. coli BL21 (DE3) cells containing the pMLBAD have a slightly higher number of CFU than *E. coli* BL21 (DE3) cells. With the stress of the high salt concentration, cells which express the Lpp antisense sequence should grow even less. As the BL21 (DE3) containing pAR2 are not more susceptible to the salt than the others, it can be said that in this case either the Lpp antisense transcript was not induced in appropriate amounts or it has low inhibiting effects on Lpp mRNA.

This test should be repeated to see if these values are representative. It should also be tried to use a higher concentration of arabinose to see if the concentration was too low and so the plasmid was not induced.

3.2.2.5 Susceptibility to SDS

This test was carried due to the same reason as explained before in section 3.2.2.4.

The average values of number from three plates of colonies from *E. coli* BL21, BL21 containing the pMLBAD vector and BL21 containing pAR2, grown under stress of SDS are listed in Table 8.

Table 8: *E. coli* cell counts (as CFU/10 µL) from test cells of susceptibility to SDS

dilution	BL21	BL21 + pMLBAD	BL21 + pAR2
10^{-4}	2.40 x 10^5	>4.00 x 10^6	1.21 x 10^6
10^{-5}	5.00 x 10^5	6.40 x10^6	1.30 x 10^6
10^{-6}	1.00 x 10^6	5.00 x 10^6	2.00 x 10^6

Similar to the test of susceptibility to salts, also the SDS test shows the highest number of colonies at BL21 (DE3) cells containing pMLBAD. The other two strains, BL21 (DE3) without plasmid and BL21 containing pAR2 should grow less due to some expected permeabilization of the cell membrane through the lack of Lpp expression motivated by the antisense oligonucleotide, but it seems that again it does not have any effect. This can be due to the reasons mentioned above, insufficient induction of the plasmid or no effect of the Lpp antisense transcript on the cells. It seems that the empty pMLBAD vector has an effect on the cells, as in both tests they grew the best. To exclude that these results are just a coincidence, more tests have to be carried out and also here it should be tried if a higher concentration of arabinose in the plates would change the results.

3.2.3 Sequencing result

The physiological tests were carried out to save some time meanwhile the plasmid was sequenced. Unfortunately, the sequencing revealed, that not the Lpp antisense sequence was cloned into the vector. Instead, a part of the *E. coli* αDH5 chromosome with approximately the same size (273 nt) was cloned into pMLBAD. This indicates that, unfortunately, there was a contamination with chromosomal DNA before the ligation. As this region is flanked by the two enzymes that were used for restriction (EcoRI and HindIII) and it does not contain the restriction site for SalI, which was used for the confirmation restriction, it was mistaken with the correct Lpp antisense region.

With this sequencing result it is obvious that the reason why all the physiological tests do not show a higher permeability of the cells when the insert is induced is the incorrect cloning of the Lpp antisense. However, after the finish of this thesis the group continued the work on this construct and cloned the correct Lpp antisense

sequence into pMLBAD. This plasmid will be used to repeat all the approaches described above.

3.3 Truncated form of OmpK 1-99 from *Vibrio alginolyticus* ATCC 17749

3.3.1 Construction of a plasmid for overproduction of the truncated form of OmpK 1-99

The aim of this experiment was to overexpress the immunogenic and hydrophilic truncated OmpK 1-99, which is part of the membrane protein that does not sit in the lipidic bilayer of the outer cell membrane. In comparison to the whole OmpK it should be more soluble and easier to express and purify.

The agarose gel with the PCR products from the amplification of the truncated OmpK 1-99 sequence can be seen in Figure 17. As the bands are approximatly at the height of the 300 bp band of the DNA ladder it can be said that the PCR product was the correct size of 316 bp.

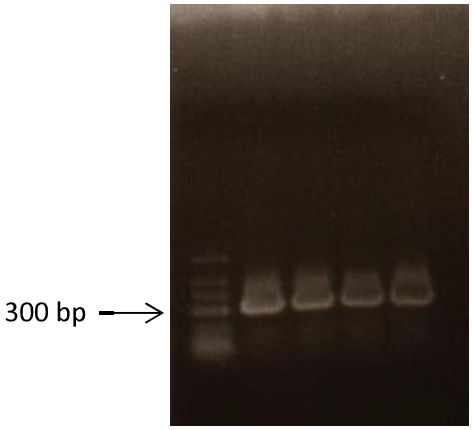

Figure 17: agarose gel; first lane - GeneRuler™ Low Range DNA Ladder; further lanes - products of four PCR batches to amplify the truncated OmpK 1-99 sequence

This truncated OmpK 1-99 sequence was ligated with pET23a+ to obtain the pAR1 vector.

The sequence of the recombinant plasmid pAR1 was obtained with Serial Cloner 1.3. This sequence and the one from pET23a+ without insert were used to draw the vectors with NEBcutter V2.0 (Vincze et al., 2003) which can be seen in Figure 18. The list of ORFs does not contains Open Reading Frames only, but general plasmid features.

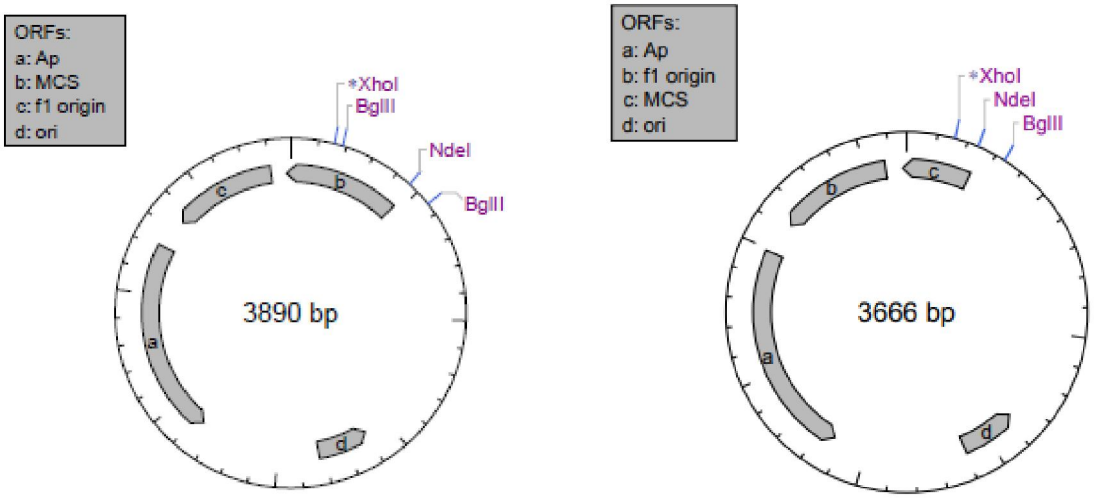

Figure 18: left - pAR1 (pET23a+ + OmpK 1-99), right - pET23a+; restriction sites of used enzymes (BglII, NdeI, XhoI) and plasmid features are indicated (Vincze et al., 2003)

When pAR1 and pET23a+ are compared it can be seen that the recombinant plasmid has an additional BglII restriction site inbetween the restriction sites for NdeI and XhoI. Due to this fact, BglII was chosen as enzyme to distinguish between pAR1 and pET23a+.

Figure 19 shows the agarose gel with the confirmation of the pET23a+ vector containing the correct truncated OmpK 1-99 peptide sequence. For comparison of the size of the fragments lane 1 shows the GeneRuler™ 1 kb DNA Ladder and lane 4 the GeneRuler™ Low Range DNA Ladder.

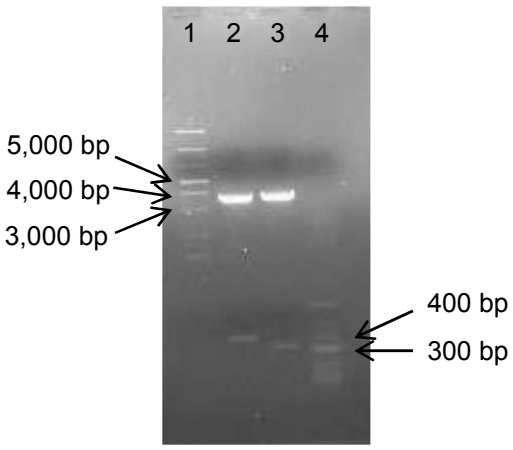

Figure 19: confirmation pET23 + truncated OmpK 1-99 sequence. Lane 1: 1 kb protein ladder. Lane 2: construct cut with BglII. Lane 3: construct cut with XhoI and NdeI. Lane 4: LR protein ladder

Lanes 1 and 4 are the DNA ladders (1 is the 1 kb and 4 is the Low Range) and lanes 2 and 3 show the construct cut with different enzymes. In lane 2 the construct was cut with BglII. In this case two bands can be seen, the bigger one with the size of 3,515 nucleotides and the smaller one with 374 nucleotides. If this transformant would have been a negative one (pET23a+) and so not containing the insert, just one band would have been seen with the size of 3666 nucleotides, because the insert has an additional restriction recognition site for the BglII enzyme, whereas the empty vector has just one site.

In lane 3 the construct was cut with the restriction enzymes used for the cloning of OmpK 1-99 insert in pET23a+, XhoI and NdeI. In this case, the two resulting fragments have the sizes of 3,586 and 316 nucleotides.

3.3.2 Truncated OmpK 1-99 overexpression optimization

To optimize the overexpression of the OmpK 1-99 protein, different approaches were tried out. As the classical overexpression was not successful, further attempts like induction at high OD_{640}, overexpression at lower temperature or also autoinduction were used.

3.3.2.1 Overexpression at 37 °C

The following Figure 20 shows the SDS-PAGE gel with the protein samples from *E. coli* BL21 (DE3) harbouring the plasmid pAR1 after overexpression under normal conditions stained with Coomassie Blue. The first lane shows the Precision Plus Protein™ Dual Xtra Prestained Protein Standard. The other lanes contain the protein samples taken during the overexpression from the cell broth representing an OD_{640} of 0.6. T0 is the protein sample taken just before the induction with IPTG and the following samples corresponding to 1 to 20 hours after induction.

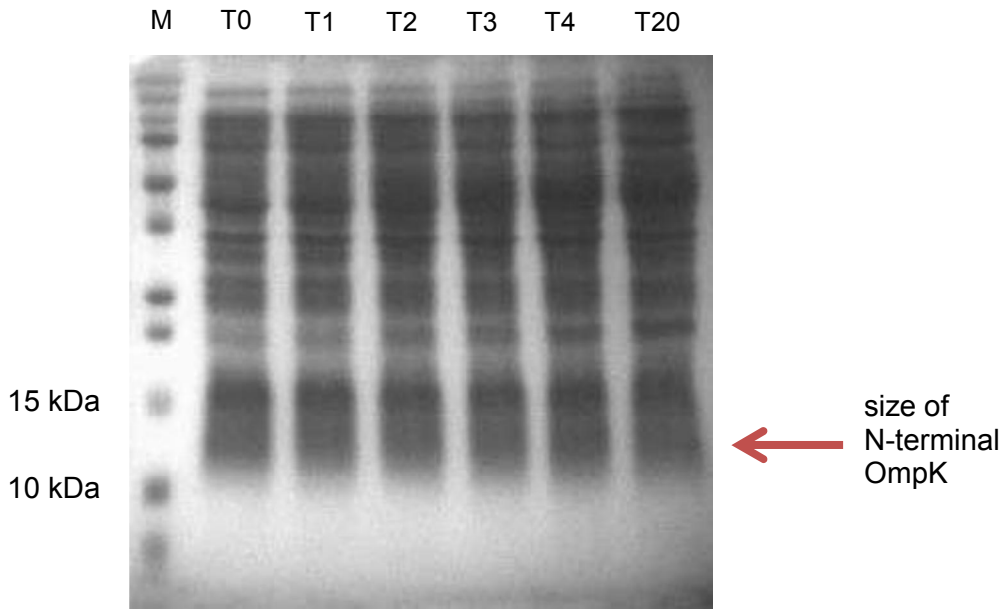

Figure 20: *E. coli* BL21 (DE3) cells containing pAR1 used for overexpression of OmpK 1-99 at 37 °C; Lane 1: protein standard. Further lanes: protein samples during overexpression 0, 1, 2, 3, 4 and 20 hours after induction

As the desired OmpK fragment has a size of 12.18 kDa, an overexpression of it should be seen in the area between the 15 kDa and 10 kDa band of the marker. Unfortunately, no increase of intensity of a protein in this area can be seen. As this area is kind of blurred and so no specific bands can be seen, also a Western-Blot analysis was carried out with these protein samples. Also the Western-Blot has not shown any signal in the desired region and so it confirmed that there was no overexpression of the truncated OmpK 1-99.

3.3.2.2 Overexpression at 37 °C with induction at high OD_{640}

The resulting stained SDS-PAGE gel from the overexpression batch induced at higher OD_{640} can be seen in Figure 21. Lane 1 represents a positive control (protein BCAL2645 from *B. cenocepacia* J2315, overexpressed from construct pSAS36 (pET23a+ with BCAL2645 gene)) and lane 2 the protein marker. The other lanes contain the protein samples immediately before the induction (T0) and up to 2 hours after it (T0.5 – T2).

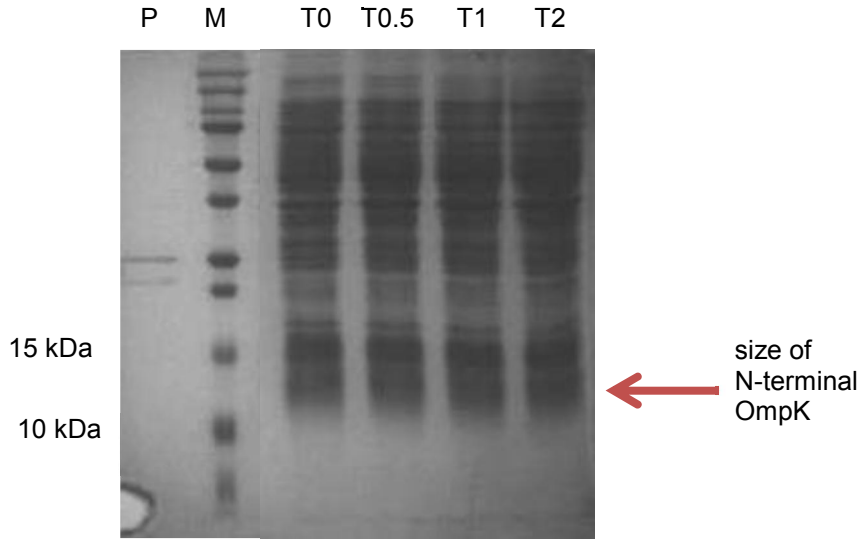

Figure 21: SDS-PAGE gel with protein samples from overexpression of OmpK fragment in transformed *E. coli* BL21 (DE3) induced at higher OD_{640}. Lane 1: positive control. Lane 2: protein standard. Further lanes: protein samples taken 0, 0.5, 1 and 2 hours after induction

Also with this attempt there is no visible overexpression of the protein in the area between 10 and 15 kDa. For further analysis a Western Blot was made, which can be seen in Figure 22. The distribution of the protein samples is the same as on the stained gel.

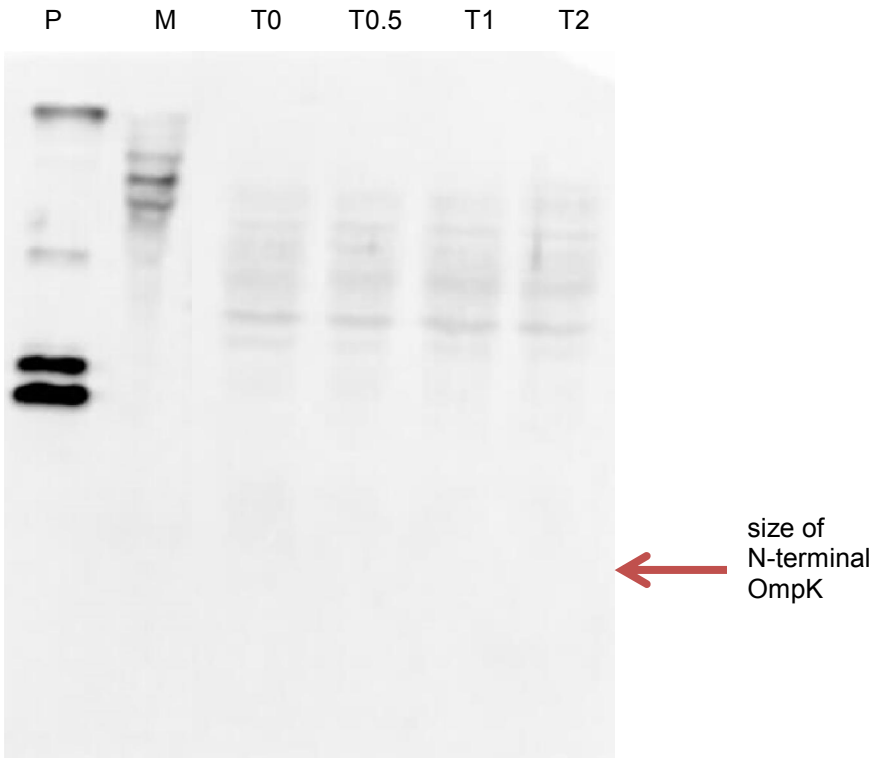

Figure 22: Western Blot with protein samples from overexpression of OmpK fragment in transformed *E. coli* BL21 (DE3) induced at higher OD_{640}. Lane 1: positive control. Lane 2: protein standard. Further lanes: protein samples taken 0, 0.5, 1 and 2 hours after induction

The two big bands are from the positive control. Basically, just overexpressed proteins with a His-tag should be seen on the Western Blot. Unfortunately, the antibody has also bound quite unspecifically to other proteins in the upper part of the membrane. In the lower part, where the desired overexpressed OmpK should be present, nothing can be seen. Therefore, it can be assumed that neither with this attempt the protein could be overexpressed.

3.3.2.3 Overexpression at 16 °C and purification

Figure 23 shows the results of the overexpression at lower temperature. The first lane shows the protein marker, the second lane the protein sample just before the induction and the third lane 16 hours after induction. The second set of samples was after IMAC purification with increasing amount of imidazole in the elution buffer to destabilize the interaction between the His-tagged peptide and metal affinity ligands in the column and, so, to elute bound protein.

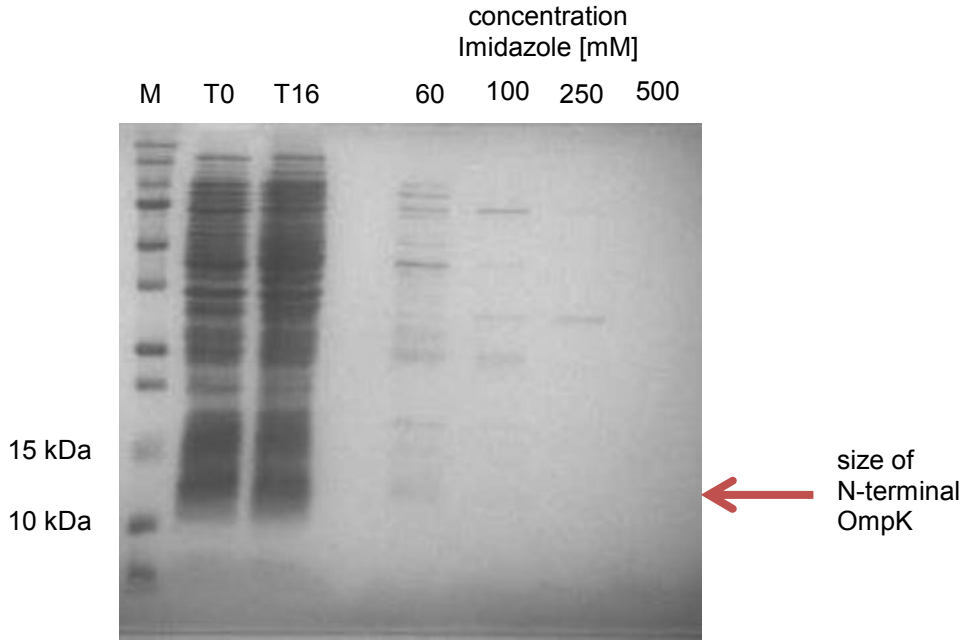

Figure 23: SDS-PAGE gel with protein samples from overexpression of OmpK fragment in transformed *E. coli* BL21 (DE3) at 16 °C and after purification. Lane 1: protein standard. Lane 2 and 3: protein samples 0 and 16 hours after induction. Further lanes: samples from the purification process

In the samples of all proteins of the cells (T0 and T16), there is no visible overexpression of OmpK. Theoretically, just the overexpressed protein is supposed to bind to the His-Trap-column at high imidazole concentrations and so the protein should be purified easily. In the samples from the purification procedure, a lot of bands can be seen. In the first purification step (60 mM imidazole) the most bands can be seen. This means that a lot of different proteins are eluted from the column, which are just weakly bound. When the concentration of imidazole is increased, fewer proteins are detected. In the last elution step there is no band visible, what means that all proteins that have bound to the column were already eluted before.

The Western Blot of these protein samples has not shown any signal from the OmpK. This indicates again that the protein was not overexpressed, even when the culture was grown at the lower temperature.

3.3.2.4 Overexpression by autoinduction

The resulting protein samples of the autoinduction trial are shown in Figure 24. Again, the first lane shows the protein marker and the lanes after, the protein samples taken during the growth. In the present study of autoinduction, the time indicates not the time after induction but after inoculation of the culture medium.

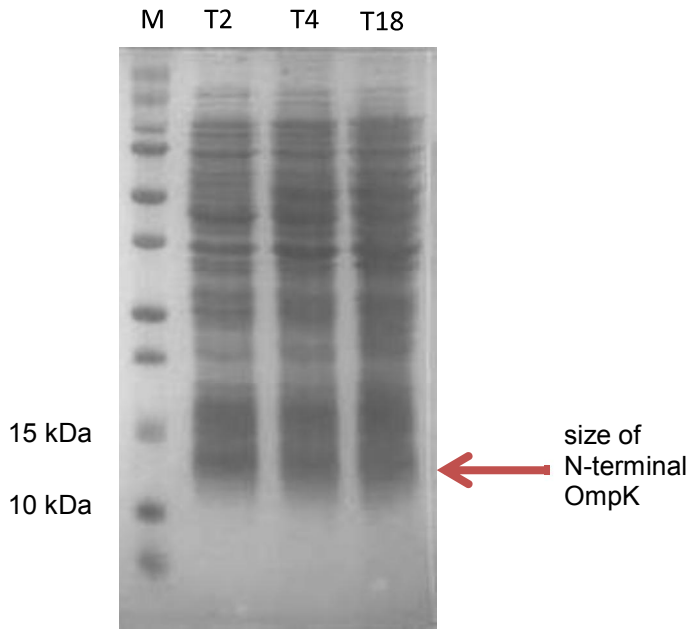

Figure 24: SDS-PAGE gel with protein samples from overexpression of OmpK fragment in transformed *E. coli* (BL21 (DE3) with autoinduction. Lane 1: protein standard. Further lanes: protein samples 2, 4 and 18 hours after inoculation

Observing the density profiles of the bands of low molecular weight in the gel (Figure 24) becomes evident that this attempt of overexpression did not show the wanted result. In the area of 12 kDa there is no visible increase of the concentration of the desired OmpK peptide over time. Furthermore, neither the Western-Blot has shown any signal in that area.

4. Conclusion and future perspectives

The chromatogram of the HIC showed that two fractions are separated efficiently. Unfortunately, as the concentration of protein was too low, it was not possible to confirm that the fractions are protein and endotoxin, respectively. This means that a bigger amount of OmpK protein has to be produced in the recombinant *E. coli* BL21 (DE3) strain containing the pJBF2 plasmid to carry out the HIC again and to check the peaks. If the SDS-PAGE shows that the protein is concentrated in one of the two peaks, the concentration of endotoxins in the respective protein pool has to be quantified. When these tests confirm, that the protein is successfully separated from the endotoxins, a higher amount of protein can be produced, purified from the endotoxins with this method and used for the production of antibodies.

During the work of this thesis it was not possible to obtain the right construct for the Lpp antisense. After this thesis was finished, the group continued work on this and was able to obtain pMLBAD with the correct Lpp antisense sequence. With this construct all of the physiological tests have to be repeated to see if it has the expected effect on the cells.

In terms of the antigenic N-terminal OmpK peptide with the natural signal peptide, the molecular manipulations were successful and so the correct construct was obtained. Unfortunately, overexpression of the peptide was not successful. One reason could be that as this peptide does not originate from *E. coli*, and as it does not fulfil any necessary task for the cell, it is degraded immediately after translation. Another reason for degradation could be that the cloned sequence of the plasmid encodes the truncated form of OmpK protein, corresponding to the 1 to 99 N-terminal amino acids region of OmpK. So, it is possible that the cells realize that it is not the complete protein and therefore try to get rid of it. Maybe more different conditions of growth can be carried out to achieve an overexpression of the peptide. If not, another attempt can be to use the C-terminal OmpK part. In this case, the sequence of the other half of the protein has to be cloned into the plasmid and with this construct all overexpression methods carried out during this thesis can be repeated to see, if this part of the protein can be overexpressed. If it is also not possible to overexpress this peptide, the whole protein has to be used. Fiúza Almeida Lopes (2017) proved in his

thesis that it is possible to overexpress the whole OmpK, but the purification is difficult. So, if it is necessary to use the whole protein, it should be tried to optimize the purification steps to produce a high amount of protein that can be used for the HIC to get rid of the endotoxins and so can be used for the production of antibodies.

5. References

AGUIRRE-GUZMAN, G., MEJIA RUIZ, H. & ASCENCIO, F., 2004. A review of extracellular virulence product of Vibrio species important in diseases of cultivated shrimp. *Aquaculture Research,* **35** (15): 1395-1404.

ÄMMÄLÄ, C., DRURY, W., KNERR, L., AHLSTEDT, I., STILLEMARK-BILLTON, P., WENNBERG-HULDT, C., ANDERSSON, E.-M., VALEUR, E., JANSSON-LÖFMARK, R. & JANZÉN, D., 2018. Targeted delivery of antisense oligonucleotides to pancreatic β-cells. *Science Advances,* **4** (10): eaat3386.

BRUNO, D. W., NOGUERA, P. A. & POPPE, T. T., 2013. *A colour atlas of salmonid diseases.* Springer Science & Business Media.

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.

CHANG, T. W., LIN, Y. M., WANG, C. F. & LIAO, Y. D., 2012. Outer membrane lipoprotein Lpp is Gram-negative bacterial cell surface receptor for cationic antimicrobial peptides. *J Biol Chem,* **287** (1): 418-28.

CLAASSENS, N. J., SILIAKUS, M. F., SPAANS, S. K., CREUTZBURG, S. C. A., NIJSSE, B., SCHAAP, P. J., QUAX, T. E. F. & VAN DER OOST, J., 2017. Improving heterologous membrane protein production in Escherichia coli by combining transcriptional tuning and codon usage algorithms. *PLoS One,* **12** (9): e0184355.

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 Biotechnol,* **25** (10): 472-9.

DIAS, N. & STEIN, C. A., 2002. Antisense Oligonucleotides: Basic Concepts and Mechanisms. *American Association for Cancer Research,* **1**: 347-355.

FIÚZA ALMEIDA LOPES, J. B., 2017. *Studies with Vibrio alginolyticus OmpK towards the development of a vaccine for Solea senegalensis.* Master of Science, Department of Bioengineering (DBE), Instituto Superior Técnico Lisboa.

FRECER, V., HO, B. & DING, J. L., 2000. Interpretation of biological activity data of bacterial endotoxins by simple molecular models of mechanism of action. *The FEBS Journal,* **267** (3): 837-852.

GACIARZ, A., KHATRI, N. K., VELEZ-SUBERBIE, M. L., SAARANEN, M. J., UCHIDA, Y., KESHAVARZ-MOORE, E. & RUDDOCK, L. W., 2017. Efficient soluble expression of disulfide bonded proteins in the cytoplasm of Escherichia coli in fed-batch fermentations on chemically defined minimal media. *Microb Cell Fact,* **16** (1): 108.

GALANOS, C., LÜDERITZ, O., RIETSCHEL, E. T., WESTPHAL, O., BRADE, H., BRADE, L., FREUDENBERG, M., SCHADE, U., IMOTO, M. & YOSHIMURA, H., 1985. Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. *The FEBS Journal,* **148** (1): 1-5.

GE HEALTHCARE BIO-SCIENCES, 2014. HiTrap™ HIC columns.

GE HEALTHCARE BIO-SCIENCES, 2018. HiTrap affinity columns (HisTrap FF, 1 ml and 5 ml).

GE HEALTHCARE LIFE SCIENCES, 2018. *Protein transfer in Western blotting* [Online]. Available: https://www.gelifesciences.com/en/bd/Solutions/Protein-Research/Knowledge-center/western-blotting/Comparison-of-transfer-methods-in-Western-blotting [Accessed 20.08.2018].

GOEMANS, N., MERCURI, E., BELOUSOVA, E., KOMAKI, H., DUBROVSKY, A., MCDONALD, C. M., KRAUS, J. E., LOURBAKOS, A., LIN, Z., CAMPION, G., WANG, S. X., CAMPBELL, C. & GROUP, D. I. S., 2018. A randomized placebo-controlled phase 3 trial of an antisense oligonucleotide, drisapersen, in Duchenne muscular dystrophy. *Neuromuscul Disord,* **28** (1): 4-15.

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**: 101-106.

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**: 245-249.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature,* **227** (5259): 680.

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.

LI, N., YANG, Z., BAI, J., FU, X., LIU, L., SHI, C. & WU, S., 2010a. A shared antigen among Vibrio species: outer membrane protein-OmpK as a versatile Vibriosis vaccine candidate in Orange-spotted grouper (Epinephelus coioides). *Fish Shellfish Immunol,* **28** (5-6): 952-6.

LI, Y. D., REN, H. L., LU, S. Y., ZHOU, Y., HAN, X., GONG, B. B., ZHANG, Y. Y. & LIU, Z. S., 2010b. Cloning, expression, and genus-specificity analysis of 28-kDa OmpK from Vibrio alginolyticus. *J Food Sci,* **75** (4): M198-203.

LI, Z., KESSLER, W., VAN DEN HEUVEL, J. & RINAS, U., 2011. Simple defined autoinduction medium for high-level recombinant protein production using T7-based Escherichia coli expression systems. *Appl Microbiol Biotechnol,* **91** (4): 1203-13.

MALIK, A., ALSENAIDY, A. M., ELROBH, M., KHAN, W., ALANAZI, M. S. & BAZZI, M. D., 2016. Optimization of expression and purification of HSPA6 protein from Camelus dromedarius in E. coli. *Saudi J Biol Sci,* **23** (3): 410-9.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J., 1982. *Molecular cloning: a laboratory manual.* Cold spring harbor laboratory Cold Spring Harbor, NY.

MORRISON, D. & ULEVITCH, R., 1978. The effects of bacterial endotoxins on host mediation systems. A review. *The American journal of pathology,* **93** (2): 526.

NI, Y., REYE, J. & CHEN, R. R., 2007. Lpp deletion as a permeabilization method. *Biotechnology and bioengineering,* **97** (6): 1347-1356.

NINGQIU, L., JUNJIE, B., SHUQIN, W., XIAOZHE, F., HAIHUA, L., XING, Y. & CUNBIN, S., 2008. An outer membrane potein, OmpK, is an effective vaccine candidate for Vibrio harveyi in Orange-spotted grouper (*Epinephelus coioides*). *Fish & Shellfish Immunology,* **25**: 829-833.

NOVAGEN, 2018a. pET-23(+).

NOVAGEN, 2018b. pET System Manual. 11 ed.

ROSANO, G. L. & CECCARELLI, E. A., 2014. Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in microbiology,* **5**: 172.

SANCHEZ-GARCIA, L., MARTIN, L., MANGUES, R., FERRER-MIRALLES, N., VAZQUEZ, E. & VILLAVERDE, A., 2016. Recombinant pharmaceuticals from microbial cells: a 2015 update. *Microb Cell Fact,* **15**: 33.

SANTOS, C. A., FERREIRA-FILHO, J. A., O'DONOVAN, A., GUPTA, V. K., TUOHY, M. G. & SOUZA, A. P., 2017. Production of a recombinant swollenin from Trichoderma harzianum in Escherichia coli and its potential synergistic role in biomass degradation. *Microb Cell Fact,* **16** (1): 83.

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. *Infect Immun,* **76** (4): 1390-409.

SHIMP, R. L., MARTIN, L. B., ZHANG, Y., HENDERSON, B. S., DUGGAN, P., MACDONALD, N. J., LEBOWITZ, J., SAUL, A. & NARUM, D. L., 2006. Production and characterization of clinical grade *Escherichia coli* derived *Plasmodium falciparum* 42 kDa merozoite surface protein 1 ($MSP1_{42}$) in the absence of an affinity tag. *Elsevier Inc.*

SILIPO, A., ERBS, G., SHINYA, T., DOW, J. M., PARRILLI, M., LANZETTA, R., SHIBUYA, N., NEWMAN, M. A. & MOLINARO, A., 2010. Glyco-conjugates as elicitors or suppressors of plant innate immunity. *Glycobiology,* **20** (4): 406-19.

SOMMERSET, I., KROSSOY, B., BIERING, E. & FROST, P., 2005. Vaccines for fish in aquaculture. *Expert Rev Vaccines,* **4** (1): 89-101.

STEIN, C. & CHENG, Y., 1993. Antisense oligonucleotides as therapeutic agents--is the bullet really magical? *Science,* **261** (5124): 1004-1012.

TERPE, K., 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.

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. *Appl Environ Microbiol,* **71** (9): 5107-15.

VINCZE, T., POSFAI, J. & ROBERTS, R. J., 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research,* **31** (13): 3688-3691.

WATANABE, N., NAGATA, T., SATOU, Y., MASUDA, S., SAITO, T., KITAGAWA, H., KOMAKI, H., TAKAGAKI, K. & TAKEDA, S., 2018. NS-065/NCNP-01: An Antisense Oligonucleotide for Potential Treatment of Exon 53 Skipping in Duchenne Muscular Dystrophy. *Mol Ther Nucleic Acids,* **13**: 442-449.

6. Annexes

Batch-based purification (Fiúza Almeida Lopes, 2017)

Denaturation of OmpK proteins from inclusion bodies for the batch-based purification

The recombinant His6-tagged OmpK protein was denatured using urea as the denaturation agent. After the sonication cell suspension were centrifuged (30 min at 12,500 x g and 4 ºC) and resuspended with 10 mL of buffer 3 (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 1 % (w/w) Triton X-100), and centrifuged again (30 min at 12,500 x g and 4 ºC), and resuspended in 10 mL of buffer 4 (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 8 M Urea) to denature the proteins. The suspensions were stirred at room temperature for 30 minutes. A solution of 1 M of DTT was added to meet 2.5 mM and stirring was maintained for 45 minutes. The suspension was then centrifuged for 30 minutes at 12,500 x g and 15 ºC, resulting in a supernatant containing the denatured protein. In order to be used in the His-tag FF columns (GE Healthcare), the concentration of urea (Sigma) was decreased from 8 M to 6 M by adding 3.3 mL of buffer 5 (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl) due to the proximity to the maximum concentration of urea allowed for the column. The suspension was stored overnight at room temperature in buffer 7 (50 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 6 M urea, 10 % glycerol)

Renaturation of denatured OmpK proteins for batch-based purification

For the renaturation process 2 mL of the denatured protein sample was added to an amount of 8mL of slurry containing 4 mL of resin of Ni sephase6FF (GE Healthcare) was used accordingly to the manufacturer's instructions centrifuged at room temperature for 5 minutes at 1,000 x g. The supernatant was discarded and 5 mL of H_2Od were added. After a 3 minutes rest at room temperature, the resin was centrifuged at room temperature for 5 minutes at 1,000 x g. The supernatant was discarded again and 5 mL of buffer 6 (10 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 6 M urea) was added. After a 3 minutes rest at room temperature, the resin was centrifuged at room temperature for 5 minutes at 1,000 x g. Resuspended in 20 mL the protein sample and incubated for one hour at room temperature and then

centrifuged at room temperature for 5 minutes at 1,000 x g. 5 mL of buffer 6 was added and incubated for 5 minutes at room temperature and centrifuged at room temperature for 5 minutes at 1,000 x g. 7.5 mL of buffer 7 was added followed by incubation for 10 minutes at room temperature. The resin was centrifuged at room temperature for 5 minutes at 1,000 x g and a sample was collected for SDS-PAGE analysis. The renaturation process started by the addition of 10 aliquots of 2 mL of buffer 8 (10 mM Tris-HCl, 0.5 M NaCl, 10 % glycerol) with decreasing concentration of urea, 0.5 decrease between each buffer addition from 6 M to 1 M. After the addition of each the slurry rested for 10 minutes and was then centrifuged at room temperature for 5 minutes at 1,000 x g before the addition of following aliquot, to ensure the drop in urea concentration. The process was continued for buffers 9 (300 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 10 % glycerol) and 10 (500 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, 10 % glycerol). The regeneration of the resin was performed as described in the manufacturer's instructions. The protein of interest pool was that from supernatant after incubation with buffer 9. These samples were dialyzed using a Slide-A-Lyzer dialysis cassette (Pierce) and placed in buffer 8 overnight at 4 °C with gentle agitation. After dialysis and to conclude the renaturation process, the sample was added to a His-tag column previously equilibrated with buffer 10. The fractions of protein that do not bind to the column were collected and should contain the renatured protein of interest. In order to collect the non-renatured and clean the column, 10 mL of buffers 10 and 5, respectively were used. Afterwards the column was stored at 4 °C.